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From the Department of Pharmacology Karolinska Institutet Stockholm Sweden

Stimulation of Gastric Acid Secretion by Irrigation of the Antrum with Some Aliphatic Alcohols[1]

By

CARL ERIC ELWIN

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Abstract

ELWIN C E *Stimulation of gastric acid secretion by irrigation of the antrum with some aliphatic alcohols* Acta physiol scand 1969 75 1—11

Ethyl alcohol is known to stimulate gastric acid secretion in fundic pouch dogs during irrigation of the antrum A comparison was made with regard to such an effect of a series of

or n propyl alcohol during simultaneous administration indicating almost no interference with gastrin release mechanisms n Propyl alcohol together with ethyl alcohol showed a dose response related action Methyl alcohol was almost inactive The results indicate that the stimulatory effect of alcohols could not be related to water or fat solubility but to the molecular configuration

Experimental studies have demonstrated that different substances stimulate gastric acid secretion on instillation into isolated antrum pouches (Robertson *et al* 1950 Woodward *et al* 1954 Woodward and Dragstedt 1960) However, very little is known about the chemical and physical properties required of such substances in order to give this effect. Ethyl alcohol has been found to be a potent stimulant of acid secretion during application in the antrum and the experimental findings indicate that the mechanism of action is by release of gastrin (Woodward *et al* 1957) Being a well defined chemical ethanol has been widely used as a stimulant to study antral stimulatory (Nyhus *et al* 1960) and inhibitory (Jordan and Sand 1957 Shapira and State 1961) mechanisms The relationship of the stimulatory effect of ethanol to variation in concentration of the compound pH and ions etc in the antrum has been studied in a recent investigation (Elwin 1969)

[1] Part of this investigation has been published in Gastrin UCLA Forum Med Sci No 5 1966 University of California Press Los Angeles

The present work was undertaken as part of a series of investigations to explore the significance of chemical properties on local gastrin release. The effect of a series of aliphatic alcohols during antrum perfusion or instillation was compared with that of ethanol to analyze the extent to which the ethanol effect is unique among alcohols.

Methods

Surgical procedures

8 adult dogs weighing between 11.5 and 19 kg were used. In another paper (see Elwin 1968) a detailed description has been given of the surgical procedures; most of the dogs appeared in both series of experiments. 7 of the animals had vagally denervated fundic pouches (nos 151, 164, 170, 179, 187, 212 and 239). In the remaining dog (no 141) a Pavlov pouch had been constructed by a technique used in our laboratory (Andersson and Uvnäs 1961). Each dog had been provided with a vagally innervated antral pouch at a second operation performed about two weeks after the first. The pyloric end of the antrum had been exteriorized after transection of the pylorus and the gastrointestinal passage restored by gastrojejunostomy. In some dogs a part of the acid-secreting mucosa occurred in the isolated antral pouches causing the pH of the spontaneous antral secretion to drop during histamine stimulation (1 mg s.c.). This was not considered significant, since all experiments were performed under standardized conditions and the pH controlled in antrum by use of buffered alcohol solutions.

Test procedures

When the experiments started in the morning the animals had been fasting for about 18 hrs. The basal secretion from the fundic pouches was recorded during at least one hr prior to antral instillation of the test solution. The gastric secretory output was collected in 15-min portions during the experiments. The volume was measured and the amount of free and total acid determined. Each portion was titrated with 0.01 N NaOH, using a mixture of Töpfer's reagent and phenolphthalein as indicator.

In all experiments a plastic cone was inserted into the antral fistula to avoid leakage from the pouches during perfusion or instillation. The outlet tube was connected to an outflow plastic tube.

Solutions of alcohols were either continuously perfused through or held in the antral pouches for different periods of time. In the perfusion experiments the alcohol solutions were administered at a rate of 100 ml per hr for 2 hrs. After passing through the antrum the test solutions had to leave the pouch through an outflow tube so arranged that an instillation pressure of 20 cm H_2O was created. In those experiments where a dose-response relationship for ethanol or n-propanol was examined, the perfusion time was limited to 30 min for each concentration of the alcohols. Secretory response was allowed to return to control level before perfusion with the next alcohol concentration. Instillation pressure of 20 cm H_2O was also used in these experiments.

In the instillation experiments 30—40 ml of alcohol solution was held in the antrum pouches for 3 hrs.

The alcohols were administered in saline and the solutions buffered to pH 3 with Mac Ilvaine's buffer[1]. The pH of the perfusate was checked regularly after the solution had passed through the antrum pouch.

The secretory response to 2 hr antral perfusion is expressed as the 3-hr acid output from the fundic pouches. Mean values of these secretory responses were also calculated as per cent of mean response obtained with 10 % ethyl alcohol solutions. Dose response relationships for the effect of ethanol and n-propanol were calculated as mean values of acid output during the two 15 min periods immediately after completion of the perfusion.

The following aliphatic alcohols were studied: Methyl, Ethyl, n-Propyl, Isopropyl, n-Butyl, Isobutyl, sec Butyl and tert Butyl Alcohol.

Results

Three series of experiments were performed involving perfusion of the antrum pouches with, respectively, one alcohol at a time, simultaneous administration of two alcohols, and administration of ethanol and n-propanol in graded doses.

[1] Composition of Mac Ilvaine's standard buffer solutions: stock solution A, 0.1 molar citric acid solution and stock solution B, 0.2 molar disodium phosphate solution.

Acid secretory response to a series of aliphatic alcohols

Five Heidenhain pouch dogs (151, 164 170 179 187) and one Pavlov pouch dog (141) were used

The isolated innervated antrum pouch was perfused for 2 hrs with alcohol solutions buffered to pH 3 Five per cent solutions were tried but not continued because secretory responses were usually small However, the resulting data were used for comparison with n Butyl alcohol and Isobutyl alcohol in the same concentration since 10 % solutions of these alcohols were not obtainable—The remaining data were obtained using 10 % solutions In the case of n Butyl alcohol and Isobutyl alcohol saturated solutions (7 9 % and 9 5 % respectively) were examined instead of 10 % solutions

Methyl alcohol—This alcohol was tested in 5 dogs (141 151 164 170 187) No stimulatory effect or only a weak one was observed The largest response occurred in dog 141 Responses varied from 0 to 39 per cent with an average of 11 per cent of the one obtained with ethanol

Ethyl alcohol—Ethyl alcohol was used as a standardizing reference in all 6 dogs As can be seen from Table I it gave a good stimulatory action in all dogs

n Propyl alcohol—All 6 dogs were used The secretory output of acid from the fundic pouches was slightly greater than for ethanol in 2 dogs (170 187) about equal in one dog (164) slightly less in 1 dog (141) and considerably less in the remaining 2 dogs (151 179) Responses varied from 48 to 115 per cent of that obtained with ethanol the average being 81 per cent

Isopropyl alcohol—Isopropanol was administered to 4 dogs In two of these (151 164) the alcohol caused almost no response In the two other dogs (179 187) however there was a clear-cut but weak stimulatory effect Acid output ranged from 2 to 43 per cent of the response to ethanol with an average of 25 per cent

n Butyl alcohol Isobutyl alcohol sec Butyl alcohol and tert Butyl alcohol — In general the butanols showed a much weaker stimulatory effect than ethanol or n propanol n Butyl alcohol was tested in 5 dogs (151 164 170 179 187) and showed only a weak effect in four The effect of isobutyl alcohol was investigated in 3 dogs (164 179 187) An effect comparable with that of n butanol was observed in two of these Sec Butyl alcohol was administered to dogs 151 164 170 179 and 187 No stimulatory effect was noticed in dog 170 A weak one was seen in dogs 164 179 and 187 Responses in the remaining dog (151) showed a moderate stimulatory action Tert Butyl alcohol was instilled in the antrum of 4 dogs (151 187 No stimulatory effect was noticed in dog 170 A weak one was seen in dogs 164 179 187) In dog 164 there was almost no stimulatory effect A weak stimulatory effect was obtained in two dogs (179 187) and an effect comparable to that of ethanol in one (151) The average acid output expressed as per cent of ethanol response was respectively 37 % for n butanol 26 % for isobutanol 34 % for sec butanol and 46 % for tert butanol

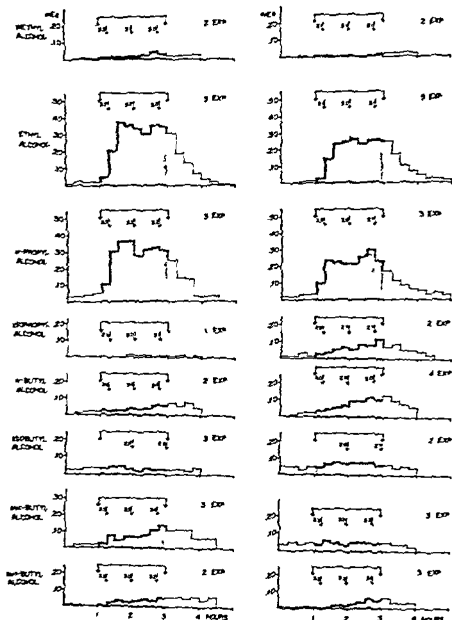


Fig 1 Secretory responses in two Heidenhain pouch dogs to irrigation of the antrum with a series of aliphatic alcohols ——— *Arrows indicate pH controls*

A complete series of experiments was performed on two dogs (164 187) This is illustrated in Fig 1 The only alcohol besides ethanol that produced in these two dogs a definite stimulation of acid secretion from the fundic pouch during antrum instillation was n propyl alcohol

Results of all experiments performed in the six dogs are summarized in Table I

DOG 164 DOG 187

Fig 2 Secretory responses in two Heidenhain pouch dogs to continuous irrigation of the antrum with a stimulatory and a "non stimulatory" alcohol
Dog 164 ⌐⊥⊥¬ = 10 % ethanol + 10 % isopropanol + 10 % ethanol
Dog 187 ⌐⊥⊥¬ = 10 % n propanol + 10 % isopropanol + 10 % n propanol

Effect of simultaneous administration of stimulatory and "non-stimulatory" aliphatic alcohols

The two stimulatory alcohols used were ethanol and n propanol, and the "non-stimulatory" alcohol was isopropyl alcohol

In 47 expts in 6 dogs (151, 164 179, 187, 212, 239) the antrum pouches were perfused either with a 10 or 16 per cent solution of the alcohol alone or with two of them mixed together in such proportions, that each compound appeared in the solution at the concentrations mentioned above Two types of experiments were performed In the first series of 9 expts in 4 dogs (151, 164 179 187) the antrum was initially perfused for one hour with 10 % ethanol or n propanol This was immediately followed by another 1-hr perfusion of a 10 % solution of isopropanol which was followed finally by another 1-hr perfusion of the initial solution, ethanol or n propanol Thus it was possible to study the influence of isopropanol on an immediately following dose of the stimulatory alcohol and this could be directly compared with the initial unaffected response in the same animal Examples of this type of experiment are shown in Fig 2 (dogs 164 187) As expected there was a decrease in the output from the fundic pouch during the second hour However no significant difference was observed in total acid output or 15 min secretory response between the initial perfusion period of the stimulatory alcohol and the one following administration of isopropanol In the second series of 38 expts in 6 dogs the antrum pouch was perfused for an initial period of 1 hr with 10 or 16 % solutions of ethanol or n propanol to provide a secretory level from the fundic pouch This was immediately followed by a 1 1/2 hr perfusion of a mixture of the stimulatory alcohol and isopropanol of the same concentration Thus the concentration of the stimulatory alcohol in the antrum was held constant during the entire experiment The results of this procedure were then compared with results from other experiments, where the antrum pouch had been perfused for a corresponding period of time with the stimulatory alcohol alone Experiments of this type are shown for dogs 164 and 212 in Fig 3 Addition of isopropanol to the perfusates apparently caused no significant change in the secretory levels of ethanol or n propanol These secretory curves and

TABLE I Total acid output from fundic pouches during antral perfusion for 2 hrs with a series of to ethanol

	Dog 141 Total acid output meq each	exp	% of EtOH	Dog 151 Total acid output meq each	exp	% of EtOH	Dog 164 Total acid output meq each	exp	% of EtOH
Methyl alcohol	0 35	0 36		0	0 01		0 33	0 18	
Mean meq	0 36		39	0 01		1	0 26		9
Ethyl alcohol	0 60	1 62		0 90	0 64		3 62	1 66	
	1 07	0 96					3 87		
	0 37								
Mean meq	0 92		100	0 77		100	3 05		100
n Propyl alcohol	1 03	0 37		0 50	0 49		2 43	3 10	
	0 56			0 12			3 07		
Mean meq	0 65		71	0 37		48	2 87		94
Isopropyl alcohol				0 04	0 18		0 06		
Mean meq				0 11		14	0 06		2
n butyl alcohol				0 42	0 10		0 64	0 40	
Mean meq				0 26		34	0 52		17
butyl alcohol							0 62	0 12	
							0 20		
Mean meq							0 31		10
sec butyl alcohol				1 19	0 34		1 57	0 86	
				0 23	0 41		0 70		
Mean meq				0 54		70	1 04		34
tert butyl alcohol				0 76	0 69		0 92	0 11	
Mean meq				0 73		95	0 52		17

those obtained with stimulatory alcohol alone were nearly identical The effect on gastric acid secretion of n propanol is very similar to that of ethanol

Effect of graded concentrations of ethanol and n-propanol

The previous findings in this investigation of the stimulatory effect on gastric secretion of a series of alcohols indicated that only one of these n propyl alcohol had an effect comparable to although slightly weaker than that of ethyl alcohol To further study this relationship the antrum pouch of two Heidenhain pouch dogs (164, 212) was perfused with varying concentrations of the two alcohols and the secretory output from the fundic pouch compared at each concentration level The secretory response of the fundic pouch occurred within 15—30 min and continued

aliphatic alcohols Secretory response expressed as 3 hr acid output and per cent of response

Dog 170 Total acid output meq each	Dog 170 exp	% of EtOH	Dog 179 Total acid output meq each	Dog 179 exp	% of EtOH	Dog 187 Total acid output meq each	Dog 187 exp	% of EtOH	Gastrin releasing potency All dogs Per cent of ethanol Mean	Range
0						0 07	0 19			
0		0				0 13		6	11	0— 39
1 23	0 67		1 69	1 84		2 30	2 59			
			1 59			2 09	2 01			
						1 12				
0 95		100	1 71		100	2 02		100	100	—
1 58	0 54		0 82	0 45		2 63	2 22			
1 15			1 22	0 99		1 79				
1 09		115	0 87		51	2 21		109	81	48—115
			0 59	0 88		0 69	0 86			
			0 74		43	0 78		39	25	2— 43
0 50	0 15		0 66	0 60		0 60	2 03			
						0 71	0 72			
						1 87				
0 31		35	0 63		37	1 19		60	37	17— 60
			0 49	0 56		0 75	0 75			
			0 53		31	0 75		37	26	10— 37
0 05			0 57	0 53		0 25	0 22			
			0 65			1 21				
0 05		5	0 58		34	0 56		28	34	5— 70
			0 39	1 40		0 42	0 46			
			0 76			0 33				
			0 85		50	0 42		21	46	17— 95

for another 30-min period 4, 8 and 16 % solutions of the two alcohols were used also in this study but have been expressed in molar concentrations to provide for a direct comparison of their potency Results are shown in Fig 4 Three experiments were performed at each concentration Higher concentrations of n propanol were not used because the toxicity is considerably greater than for ethanol Even with ethanol it was found that higher concentrations tended to cause a decrease in secretory output A clear dose response relationship for n propanol was demonstrated within the concentration range tested In one dog (164) the stimulatory potency of n propanol was found to be slightly less than for ethanol at the concentrations used whereas in the other dog (212) it was about the same The curves for the acid output from the fundic pouches were nearly alike in the two dogs

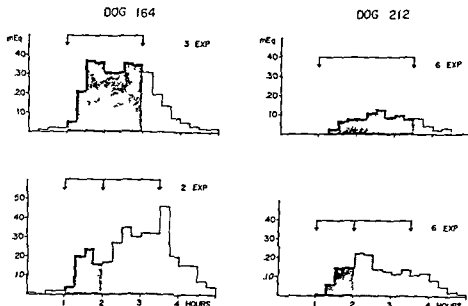


Fig 3 Secretory responses in two Heidenhain pouch dogs to irrigation of the antrum with a stimulatory and a non stimulatory alcohol

↓———↓ = ethanol
↓—↓—↓ = ethanol + (ethanol and isopropanol)
164 10 % solutions buffered to pH 3
212 16 % solutions buffered to pH 3

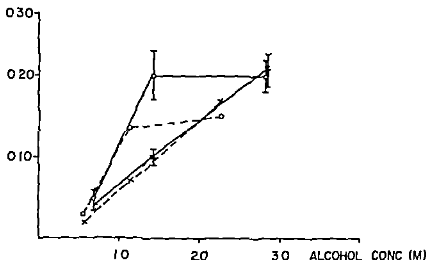


Fig 4 Dose response relationship for stimulation of gastric acid secretion by irrigation of the antrum with ethanol and n propanol

○——○ = Dog 164 ethanol ———× Dog 212 ethanol
○ ○ = Dog 164 n propanol × × Dog 212 n propanol

Discussion

Ethyl alcohol is the only alcohol previously shown to stimulate gastric acid secretion The effect of ethanol after oral ingestion is mainly due to two factors a direct action on the oxyntic glands after absorption, and an indirect action by release of gastrin after local contact with the antrum mucosa (Woodward *et al* 1957, Hirschowitz *et al* 1956, Weise, Schapiro and Woodward 1961) Woodward *et al* (1957) demonstrated that antrum perfusion with ethanol solutions initiates a pronounced acid output from denervated fundic pouches without any measurable increase of the blood alcohol concentration From their experimental data it was concluded that the only propable mechanism for ethanol to stimulate gastric acid secretion during antrum instillation was by release of gastrin Similar conclusions have been reached by other investigators (Irvine *et al* 1960), and no data have yet been presented to contradict this hypothesis

The final role of ethanol in gastrin release may involve a complex pattern Other aliphatic alcohols show biological properties similar to those of ethanol (Gage 1965) It was therefore considered of interest to compare the stimulatory effects of a series of closely related aliphatic alcohols This might yield valuable information of the general features of antral stimulation by small molecules of a certain type and at the same time reveal the specificity of the effect of ethyl alcohol

In a recent study (Elwin 1969) it was found that the concentration of the test solution, hydrogen ions and certain other ions of physiological importance, influence the rate of gastrin release by ethanol It is possible that variations in the milieu interfere with the penetration through the mucosa to the receptor organ The chemical and physical properties of the test substance itself may also be of fundamental importance in this respect For instance, little is known concerning the significance of such physical properties as water and fat solubility to the potency of chemical secretagogues in antrum

In this investigation the effects of 8 aliphatic alcohols were examined in relation to acid output from fundic pouches during antrum instillation of the compounds Only two of the alcohols ethanol and n propanol showed a potent stimulatory effect All other alcohols tested had an effect less than half of that of ethanol It is known that the water solubility varies between the alcohols and this difference could be one reason for their varying stimulatory effects Methanol ethanol n-propanol and iso-propanol are all miscible with water in any proportion but only ethanol and n propanol had a stimulatory action Methanol and iso-propanol showed only a slight stimulatory effect or none at all in most dogs Furthermore water solubility increases from n butyl alcohol to tert butyl alcohol The solubility of n butyl alcohol is very low (7 9 % in 100 ml H_2O at 20° C) whereas tert butyl alcohol shows the same solubility in water as ethanol or n propanol All the butyl alcohols had a much weaker stimulatory effect on gastric secretion than either ethanol or n-propanol Thus it seems unlikely that the stimulatory effect of alcohols is related to their water solubility

All the alcohols used in these experiments are fat soluble Fat solubility increases with the length of the alcohol's carbon chain The butanols are more readily fat soluble than, for instance methanol and ethanol It might have been concluded, from the action of the three alcohols methanol, ethanol and n propanol, that the relatively greater stimulatory effects of ethanol and n propanol were due to their higher degree of fat solubility The butanols should then be expected to have an even greater effect than ethanol and n propanol This was not the case Fat solubility is apparently not an important factor in explaining the specific stimulatory effects of the alcohols

Since neither water solubility nor fat solubility can explain the difference other explanations must be sought At present, it may be suggested that stimulatory action is determined by the molecular configuration The fact that ethanol and n-propanol have about the same stimulatory effect indicates that the action is limited to alcohols with 2 or 3 carbon atoms (see Table I) The essential chemical configuration seems

```
       H  H           H  H  H
to be  H—C—C—OH  or  H—C—C—C—OH
       H  H           H  H  H
```

The effect decreases when the length of the carbon chain is shortened to one carbon atom or lengthened to four carbon atoms Furthermore, a rearrangement of the OH group in the n-propanol molecule, changing it to the isocompound

```
CH3 H
   \
    C—OH
   /
CH3
```

causes a marked decrease of the stimulatory action This finding, combined with the appearance of a dose-response relationship for the stimulatory effect of both compounds, points to a specific action on some small chemical receptor located somewhere in the antrum mucosa A similar structure action relationship exists among the amino acids (Elwin, to be published)

The alcohols with no, or only a weak stimulatory effect on gastric acid secretion exerted neither inhibitory nor synergistic effects on the activity of the stimulatory alcohols during simultaneous administration (Fig 3) This again favors the interpretation that the activity of the alcohol is bound to small molecules of specific chemical configuration

A wealth of data indicate that ethanol stimulates gastric acid secretion, after antrum instillation by release of gastrin (*e g* Woodward *et al* 1957) The present results show that n propanol also stimulates acid production from the fundic pouch during application to the antrum In fact n propanol is almost as strong a stimulant as ethanol (Fig 4) In the different experiments performed, the secretory pattern after administration of n propanol was similar to that of ethanol It therefore seems likely that the stimulatory action of n propanol is also by release of gastrin

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Some Factors Influencing the Stimulatory Effect of Ethanol on Gastric Acid Secretion during Antrum Application[1]

By

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Abstract

ELWIN C E *Some factors influencing the stimulatory effect of ethanol on gastric acid secretion during antrum application* Acta physiol scand 1968 *75* 12—27

Variations in gastric secretory response to antral application of ethanol were studied in Heidenhain and Pavlov pouch dogs with isolated innervated antral pouch to disclose the influence of instillation pressure temperature concentration pH and ion composition Secretory response increased in 2 of 5 dogs and remained unchanged in 3 dogs during rise of installation pressure from 0 cm H_2O to 20—30 cm H_2O Secretory responses were essentially alike whether alcohol solutions were administered at room temperature or at body temperature Threshold stimulatory concentration was 2—4 % (v/v) optimal effect occurred at 8—16 % a decrease was usually observed at 32 % Antrum pouches were perfused with buffered ethanol solutions at pH values between 7 0 and 1 0 No significant difference of the stimulatory effect was noted within the pH range 7 0—3 0 At pH 2 0 the stimulatory action was decreased by about 40 % and at pH 1 0 almost totally suppressed The slope of the pH curve seems to be independent of the ethanol concentration The pH curves for the stimulatory effect of alcohol in the antrum and for vagus stimulation are almost identical The ethanol effect was inhibited by high concentrations of K^+ and Ca^{++} but uneffected by corresponding concentrations of Na^+ and Mg^{++}

It has been known since the end of the last century (Chittenden Mendel and Jackson 1898) that the ingestion of ethanol stimulates gastric secretion The stimulatory mechanism is not entirely clear but several studies suggest that at least two main paths are involved

It was suggested early that ethanol after absorption stimulates the oxyntic glands This hypothesis was supported by the fact that intravenous injection of ethanol produced gastric secretion Furthermore a linear relationship seems to exist between blood alcohol level and the secretory response (Weise Shapiro and Woodward 1961) The mechanism by which blood alcohol stimulates the oxyntic glands is not known

In 1957 Woodward *et al* showed that introduction of alcohol into an isolated antral pouch stimulated acid secretion whereas introduction into a fundic pouch was

[1] Part of this investigation has been published in Gastrin UCLA Forum Med Sci No 5 1966 University of California Press, Los Angeles and in Postgraduate gastroenterology Proceedings of a conference, Glasgow, Dec 1 3 1965 Baillière Tindall & Cassell 1966

without effect This finding suggested that alcohol liberates gastrin from the antral mucosa Measurements of blood alcohol concentration disclosed some absorption through the fundic and duodenal mucosa but no alcohol could be detected in the peripheral blood after perfusion of the antral pouches However, ethanol is absorbed from an isolated antrum pouch (Elwin, to be published) but the concentration of ethanol in the peripheral blood will apparently be too low to stimulate the oxyntic glands directly

The gastrin releasing potency of alcohol is influenced by acidity Lowering the pH of the antral contents during alcohol perfusion suppresses the stimulatory action of the alcohol (Woodward *et al* 1957, Nyhus *et al* 1960 Olbe and Elwin 1965)

The present investigation was undertaken to study the influence of factors such as concentration, pH temperature and ion composition which may effect the gastrin-releasing potency of ethanol

Methods

Surgical procedures

The experiments were performed on 13 mongrel dogs weighing 10—19 kg At an initial operation 9 dogs were provided with vagally denervated fundic pouches (Heidenhain type) Pavlov pouches were constructed in the remaining 4 dogs by a technique used in our laboratory About two weeks after the first operation vagally innervated antral pouches were constructed in the following way
oral to the presumpti
between the antrum
structed according to
method was used to localize the antrum corpus border (Olbe 1963) The pylorus was transected and the duodenum closed The pyloric end of the antrum was brought through the abdominal wall as a cutaneous fistula The gastrointestinal passage was restored by gastrojejunostomy The mode of preparation is shown in Fig 1

Test procedures

Food was removed from the cages about 18 hrs before each test The basal secretion from the fundic pouches was recorded for a control period of one to 2 hrs prior to the experiment

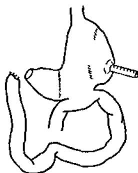


Fig 1

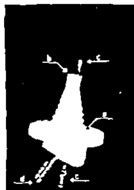


Fig 2

Fig 1 Heidenhain (H) or Pavlov (P) pouch with isolated innervated antrum pouch and gastrojejunostomy

Fig 2 Plastic cone for insertion through the cutaneous fistula into the antrum pouch
a — diameter at the base 19 mm b = diameter at the top exterior 7 mm interior 5 mm
c = inlet cannula interior diameter 1 5 mm d = outlet tube interior diameter 4 mm

Samples of gastric juice were collected at 15 min intervals during the experiments The volume was measured and the amount of free and total acid determined by titration with 0 01 N NaOH, using a mixture of Topfer s reagent and phenolphthalein as indicator

pH of the antral secretion was checked in at least 1 expt on each dog during histamine stimulation (histamine dihydrochloride, 1 mg s c) In some dogs in which the older method had been used for construction of the antral mucosal wall, a pH of 2—3 was found during histamine stimulation indicating that some acid producing fundic mucosa remained in the antrum pouches. Corpus mucosa was not found in antral pouches, where the antrum-corpus border had been visualized by means of pH indicator during surgery Solutions of alcohol were either perfused through or placed in the antral pouches for different periods of time The alcohol solutions were administered at a rate of 100 ml per hr during the perfusion experiments Leakage from the antral pouches was avoided by inserting a plastic cone into the antral fistula (Fig 2 The inlet tube passed through the cone to the bottom of the antral pouch. The outlet tube was wider and was bent at the base to allow passage of the inlet tube straight forward The outlet tube was then connected to the plastic outflow tube

Pressures

10—20 ml of the test solution was placed in the antral pouch and held there for 3 hrs In these cases the outflow tube from the antrum was connected to a vertical, open-ended glass tube about 100 cm in length in which the instilled solution could move freely Pressures are expressed as cm H_2O measured from the same horizontal level as the estimated deepest part of the antral pouch Pressure was increased by adding liquid paraffin into the vertical glass tube and correction was made for the difference in specific gravity between liquid paraffin and water Comparisons were made between ethanol solutions saline and Mac Ilvaine s buffer[1] In all perfusion experiments the outflow pressure was held at 20 cm H_2O

Temperature

The inflow tube to the antrum pouch was passed through a jacket of circulating water at controlled temperature By arranging two thermometers in the near vicinity of the entrance of the antrum one within the outflow catheter the temperature of the test solution could be measured immediately before and after passage through the antrum.

pH

In a first series, antral pouches were perfused with ethanol in normal saline and distilled water without buffering The pH of the solutions was determined before instillation and checked in samples from the effluent solutions every 15 min.

In a second series the antrum was perfused with ethanol in Mac Ilvaine s buffer or in solutions of HCl in order to hold the antrum pH constant.

Ionic composition and strength

Various amounts of NaCl, KCl $CaCl_2$ and $MgCl_2$ were added to the alcohol solutions. Comparisons were also made between isotonic saline and demineralized water

Results

Influence of instillation pressure on gastrin release by ethanol

5 Heidenhain pouch dogs were used 10 and 16 % ethanol solutions in Mac Ilvaine s buffer were instilled into the isolated antral pouch and held there for 3 hrs Saline or buffer alone was used as control The volume of the instilled solutions varied between 10 and 20 ml The secretory output from the fundic pouch was determined for saline or buffer in the antrum pouches of 5 dogs at pressures of 0 20 and 30 cm H_2O and compared with the effect of ethanol at corresponding pressures (Table I) Two different ethanol concentrations were used in one dog (239) In 2 dogs (151, 164) there was a definite increase in the secretory response to ethanol when the pressure in antrum was changed from 0 to 20 or 30 cm H_2O In the other 3 dogs (212, 237, 239)

[1] Composition of Mac Ilvaine s standard buffer solutions stock solution A 0 1 molar citric acid solution and stock solution B 0 2 molar disodium phosphate solution

TABLE I Secretory response in Heidenhain pouch dogs to antrum instillation of ethanol with and without distension Saline is used as control

		Total acid output meq/3 hrs () = number of expts mean ± SEM	Instillation pressure cm H_2O
Dog 151			
	Saline (10 ml)	0 25 (2)	20
	10 % EtOH (10 ml)	0 38 ± 0 14 (3)	0
	10 % EtOH (10 ml)	0 75 ± 0 05 (5)	20
Dog 164			
	Saline (15 ml)	0 13 (2)	30
	10 % EtOH (15 ml)	0 57 (1)	0
	10 % EtOH (15 ml)	0 90 (2)	30
Dog 212			
	Saline (15 ml)	0 02 (1)	20
	16 % EtOH (15 ml)	0 42 (2)	0
	16 % EtOH (15 ml)	0 31 ± 0 03 (3)	20
Dog 237			
	Buffer pH 5 (20 ml)	0 21 ± 0 07 (4)	20
	10 % EtOH (20 ml)	0 88 ± 0 15 (5)	0
	10 % EtOH (20 ml)	1 00 ± 0 16 (7)	20
Dog 239			
	10 % EtOH (20 ml)	0 43 (2)	0
	10 % EtOH (20 ml)	0 30 (1)	20
	16 % EtOH (20 ml)	1 30 (1)	0
	16 % EtOH (20 ml)	0 90 ± 0 10 (4)	20

the secretory response remained almost unchanged as antral pressure was increased The increase in secretory output from the fundic pouch almost always appeared during the first 1—1 1/2 hr of the instillation period as shown in Fig 3 (dog 164)

To detect release of subthreshold amounts of gastrin produced by distension the following test was made In 5 tests on two dogs (151 212) a stable choline ester (Betacholyl) was administered i v (20 μg per hr) for 4 1/2 hrs in amounts causing submaximal responses from the fundic pouch When the response of the fundic pouch had reached a constant level buffer solutions of pH 3 were instilled into the antral pouch at a pressure of 20 cm H_2O and kept there for 1 1/2 hr Clearly, the pressures applied to the antrum did not produce any further secretory stimulation Apparently the distension pressure applied to the antrum pouch in these experiments did not cause the release of measurable amounts of gastrin

Influence of temperature of the test solution on gastrin release by ethanol

In 2 dogs (141 164) the antrum was perfused with 10 % ethanol solutions within the following temperature ranges 10—20° C 20—25° C 35—40° C and 45—55°

TABLE III Acid output (meq per 15 min, mean and SEM) during two 15 min periods following 30 min perfusion of antrum with different ethanol concentration in Heidenhain (H) and Pavlov (P) pouch dogs

Ethanol concentrations (in per cent v/v)	1		2		4		8		16		32	
Dog no	Exp	meq/ 15 min	Exp	meq/ 15 min	Exp	meq/ 15 min	Exp	meq/ 15 min	Exp	meq/ 15 min	Exp	meq/ 15 min
151 (H)	1	0 05	1	0 06	5	0 14 ±0 03	5	0 18 ±0 02	5	0 08 0 02	2	0 01
164 (H)	—	—	—	—	5	0 05 ±0 01	5	0 20 ±0 03	7	0 20 ±0 02	2	0 20
187 (H)	1	0 01	1	0 01	3	0 03	5	0 17 ±0 03	5	0 31 ±0 03	4	0 16 ±0 02
212 (H)	—	—	—	—	4	0 04 ±0 01	4	0 10 ±0 01	5	0 21 ±0 02	1	0 09
335 (P)	—	—	1	0 03	3	0 04	3	0 16	3	0 47	2	0 29
460 (P)	—	—	1	0 02	1	0 00	1	0 02	1	0 18	1	0 33

histamine stimulation The importance of oxyntic glands left in the antrum is further illustrated in Fig 5 The pH of the effluent alcohol solution was already decreased within 15 min and stayed low during the entire experiments Furthermore it is shown that the pH-change was not the same in all experiments It follows from these experiments that buffered solutions must be used under such circumstances

In three Heidenhain pouch dogs and two Pavlov pouch dogs the antrum was perfused with buffered ethanol solutions at pH values between 7 0 and 0 9 or 1 0 Each experiment was performed with the alcohol solution at a constant pH 2—6 expts were done at each pH level The perfusion period was two hours Secretory response is expressed as the acid output in meq HCl during 2 1/2—3 hrs The concentration of alcohol was chosen which would produce a maximal response from the fundic

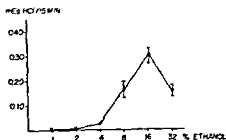


Fig 4 Dose-response relationship for antral gastrin release by different concentrations (v/v) of ethanol (dog 187) Each point represents the mean of 1—5 expts The vertical bars indicate the SEM

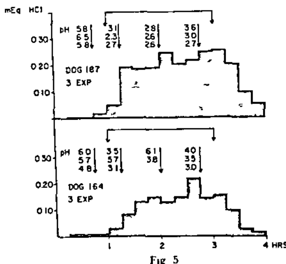


Fig 5

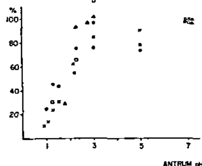


Fig 6

Fig 5 Changes of pH of ethanol solutions in distilled water (dog 164) or saline (dog 187) during antrum perfusion (↓——↓) Arrows indicate time when pH was measured pH at arrows before start of perfus on indicate pH of original ethanol solutions

P 1 (P nd He denha n (H) po ch dogs as percentage

pouches However, some of these experiments were started before the dose response curves were known for some individual dogs In spite of this the concentrations can be considered as optimal for each dog Fig 6 shows that the stimulatory action of alcohol is decreased by about 40 % at around pH 2 0 At pH 1 0—1 5 the response was almost totally suppressed Detailed data for three Heidenhain pouch dogs are presented in Table IV The appearance of the pH curves for dogs with Heidenhain pouches or Pavlov pouches were not found to differ significantly

In one Heidenhain pouch dog a comparison was made of the pH curves for 5 and 10 % ethanol (Table V) 5 % ethanol is just above the threshold concentration in this dog (Table III) and 10 % is the optimal concentration The pH curves for the two solutions were the same (Fig 6) The secretory response to 5 % ethanol at pH 3 was 118 % of the response at pH 7 The response to 10 % ethanol at pH 3 was reduced to 85 % of the response at pH 7 At pH 2 0—2 25 the responses were 67 and 63 % respectively and at pH 1 1 32 and 24 % for the respective alcohol solutions

Influence of some cations on gastrin release by ethanol

The effect was investigated in 4 series of experiments of different concentrations of sodium potassium calcium and magnesium ions on gastrin release by ethanol The chloride salts were used

TABLE IV Secretory responses in 3 Heidenhain pouch dogs to perfusion of antrum with ethanol of different acidity

	Number of expts	Total acid output meq each exp			Mean value meq	Mean value pH	Percentage secretory response pH 7=100
Dog 164	6	3 21	1 89	2 44	2 74	7 0	100
10 % ethanol		2 84	4 17	1 93			
(v/v)							
	3	2 75	2 33	2 71	2 59	5 0	91 2
	6	2 22	2 39	2 65	2 34	3 0	85 4
		1 91	2 03	2 85			
	4	1 26	1 68	1 89	1 73	2 0	63 1
		2 09					
	3	0 63	1 02	0 92	0 86	1 5	31 2
	4	0 97	0 46	0 55	0 66	1 2—1.3	24 1
		0 66					
	3	0 44	0 41	0 32	0 39	1 0—1 1	14 2
	2	0 34	0 29		0 31	0 9	11 5
Dog 212	3	1 37	1 88	1 62	1 62	7 0	100
16 % ethanol	4	1 74	1 50	0 78	1 29	5 0	79 6
(v/v)		1 15					
	3	1 42	1 44	0 87	1 24	3 0	76 5
	3	1 18	1 65	0 84	1 22	2 0—2.5	75 3
	4	0 95	0 44	0 74	0 72	1.5	44 4
		0 77					
	3	0 80	0 87	0 55	0 74	1 0—1 2	45 6
	2	0 29	0 13		0 21	1 0	12 9
Dog 239	2	2 01	1 13		1 57	7 0	100
16 % ethanol	3	0 82	1 47	1 21	1 17	5 0—5 25	74 5
(v/v)	6	0 87	1 04	1 45	1 55	3 25	98 7
		1 97	2 49	1 53			
	2	0 79	0 95		0 87	2 1—2 4	55 4

1 *Comparison between distilled water and isotonic solutions of NaCl*

The antrum pouches of 2 dogs (151, 164) were perfused for 2 hrs (100 ml per hr) with 10 % ethanol in demineralized distilled water or 0 15 n NaCl. The comparative data are based on acid output during 3 hrs including the 2 hrs of perfusion. In dog 151 the mean secretory response of the fundic pouch to antrum instillation of ethanol in distilled water was 0 98 meq (2 expts) and to ethanol in isotonic NaCl 0 91 meq (4 expts). The corresponding figures for dog 164 were 1 76 meq (distilled water 2 expts) and 2 91 meq (0 15 n NaCl 3 expts). Absence of sodium or chloride ions in the test solution did not significantly influence the effect of ethanol on gastrin release

2 *Hypotonic isotonic and hypertonic solutions of NaCl*

Six dogs were used in this series and the same experimental procedure was followed as above (1)

In 5 dogs (Table VI) a comparison was made between 0 15 n (0 9 %) and 0 85 n (5 %) NaCl as vehicles No difference in acid secretory response due to antrally applied ethanol was observed in three dogs (141 151, 164) with the two vehicles In one dog (179) the use of 0 85 n NaCl as vehicle initiated a considerably more pronounced secretory response than did 0 15 n NaCl whereas in another dog (170) the effect of ethanol in 0 85 n NaCl was less than in 0 15 n

In another comparative study on 2 dogs (151, 180) the antrum pouches were perfused with ethanol in 0 085 n (0 5 %) 0 85 n (5 %) and 1 7 n (10 %) NaCl Mean acid output for dog 151 was 1 32 meq (2 expts) 1 29 meq (3 expts) and 2 01 meq (1 expt), respectively In dog 180 the corresponding figures were 1 11 meq (3 expts), 0 95 meq (5 expts) and 1 82 meq (1 expt) Both experiments in which 1 7 n NaCl was used showed a higher acid output than the experiments performed with 0 85 n NaCl These findings indicate that strong hypertonic solutions of NaCl tend to facilitate the ethanol effect on gastrin release and that high concentrations of these ions do not exert any inhibtory effect on the action of this stimulant

3 *Comparison between isotonic solutions of KCl and NaCl*

These tests were made on the same dogs and in the same way as described above The results obtained with ethanol in saline in earlier experiments were used as reference standards The antrum pouches in 5 dogs (141 151 164 170 179) were perfused with 10 % ethanol in 0 15 n KCl in 26 expts The mean secretory output from the fundic pouch in each dog was not significantly different from the mean secretory output for ethanol in 0 15 n NaCl

4 *Comparison between solutions of KCl CaCl and MgCl₂ of varying osmolarity*

Seven dogs were used in these experiments which were with a few exceptions performed in the way mentioned above The test solutions consisted of 10 % ethanol in 0 15 n and 0 85 n KCl CaCl and MgCl In some experiments continuous irrigation of the antrum pouch with the vehicle alone was started 1 hr before instillation of the test agent ethanol and was finished 1 hr later

In three dogs the mean 3 hr acid output with 10 % ethanol in respectively 0 15 n and 0 85 n KCl was 1 70 meq and 0 84 meq (dog 141) 2 94 meq and 0 34 meq (dog 164) and 0 77 meq and 0 35 meq (dog 179) These results are based upon 6 and 2 6 and 5 4 and 2 expts respectively No stimulation of the secretory output was observed with 0 85 n KCl alone in 9 such experiments but there was a tendency to cause nausea and emesis Experiments were also conducted on 2 dogs (164 212) to provide a comparison between continuous and single dose administration of ethanol in high concentrations of KCl Results for dog 164 are shown in Fig 7 which also gives the data for corresponding experiments with isotonic KCl Antrum perfusion with 0 85 n KCl solution almost completely inhibited the release of gastrin by

TABLE V Acid secretory response in a Heidenhain pouch dog (164) during antrum perfusion of 5

5 % (v/v) Ethanol

Number of expts	Total acid output meq each exp			Mean value meq	Percentage secretory response pH 7 = 100	pH
2	1 73	1 33		1 53	100	7 0
3	2 44	1 49	1 47	1 80	117 8	3 0
2	0 85	1 18		1 02	66 6	2 0—2 25
2	0 54	0 44		0 49	32 0	1 1

ethanol Antrum instillation with a single dose of 0 85 n KCl had no effect on gastrin release under these experimental conditions

The effect of 0 15 n solutions of CaCl on release of gastrin by 10 % ethanol was studied in 10 expts on 4 dogs (141 151 164 180) Antrum perfusions were maintained for 2 hrs The 3 hr acid output indicate no significant difference from that obtained using ethanol in isotonic NaCl but was found to be somewhat higher High concentrations of $CaCl_2$ (0 85 n) in 10 % ethanol solutions caused a pronounced decrease in the secretory responses obtained in 7 expts on 3 dogs (141 164 180) but the effect of ethanol on gastrin release was on no occasion completely inhibited

With these findings in mind it was of interest to investigate the effect of another divalent cation such as magnesium No differences could be shown in the acid output from the fundic pouch in 2 dogs (179 180) using 0 15 n (7 expts) or 0 85 n (6 expts) solutions of MgCl The secretory responses were of the same magnitude as with ethanol in saline

Discussion

Ethyl alcohol was known to stimulate gastric secretion a long time before its effect on gastrin release was recognized and was therefore commonly used as a test meal Later on, when the powerful gastrin releasing action of ethanol in direct contact with the antrum mucosa became known it was used more extensively as an experimental tool to study mechanisms for local gastrin release (Nyhus *et al* 1960) and inhibition (Jordan and Sand 1957) In studies on the action of a chemical agent causing gastrin release it is important to use standardized experimental conditions Knowledge of the mechanism by which alcohol releases gastrin is still limited The purpose of the present study was to investigate how the experimental conditions should be standardized with regard to antral distension temperature concentration of test agent pH

or 10 % ethanol solutions of different pH as percentage of the response at pH 7

10 % (v/v) Ethanol						
Number of expts	Total acid output meq each exp			Mean value meq	Percentage secretory response pH 7 = 100	pH
6	3 21	2 84	1 89	2 74	100	7 0
	4 17	2 44	1 93			
6	2 22	1 91	2 39	2 34	85 4	3 0
	2 03	2 65	2 85			
4	1 26	1 89	1 68	1 73	63 1	2 0—2 25
	2 09					
4	0 97	0 55	0 46	0 66	24 1	1 1
	0 66					

and ion composition Some data on the influence of extreme variations in these factors are also presented

It seems to be important to distend the isolated antrum pouch to a certain degree during perfusion or instillation of a chemical in order to bring the test agent into close contact with the target structures In two out of five Heidenhain pouch dogs the secretory response from the fundic pouch (Table I) increased during antrum instillation of 10 % or 16 % ethanol solutions when distension was added and antrum pressure increased from 0 cm H_2O to 20 or 30 cm H_2O This is in accordance with a finding by Andersson and Olbe (1964) that in order to elicit optimal effects of acid inhibition on vagal gastrin release in some dogs with Pavlov pouches it was necessary to acidify the innervated antrum against a small outflow pressure (about 10 cm

TABLE VI Acid secretory response in fundic pouch dogs to antrum irrigation with 10 % (v/v) ethanol in isotonic or hypertonic solutions of NaCl

Dog no	Total acid output meq/3 hrs () = Number of expts Mean	
	0 15 n NaCl	0 85 n NaCl
141	0 98 (5)	0 97 (2)
151	0 87 (1)	1 29 (3)
164	2 91 (3	2 05 (3)
170	0 86 (4)	0 27 (1)
179	0 37 (1)	0 74 (2)

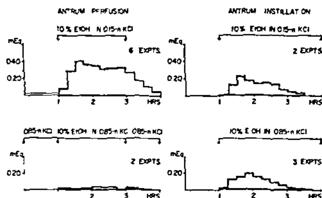


Fig 7 Influence on gastrin release by ethanol of 0 15 n and 0 85 n KCl in a Heidenhain pouch dog (164) The antrum pouch was either perfused for 2 hrs with the ethanol solution or 30 ml of the ethanol solution was held in the pouch for 3 hrs Distension pressure = 20 cm H_2O in both types of experiments

H_2O) In the experiments reported in the present study, distension itself to a pressure of 20 to 30 cm H_2O within the innervated antrum has apparently not caused mechanical stimulation with liberation of measurable amounts of gastrin, since the secretory output from the vagally denervated fundic pouch did not increase after antrum instillation of buffer and increasing the sensitivity of the fundic pouch by continuous intravenous administration of a cholinomimetic agent It has also been shown in motility studies (Olbe and Jacobson 1963) that antral type II waves of magnitude greater than 10 cm H_2O do not cause acid secretion in Pavlov pouch dogs However, in other experiments it has been shown that distension at 25 cm of pressure at a neutral pH causes release of gastrin both before and after antroneurolysis in dogs provided with vagally denervated fundic pouches (Nyhus *et al* 1960) The varying results from different sources are perhaps to be referred to differences in surgical preparation differences in the technique of applying distension and to differences of pH The test solution of the slightly distended antrum in the present study could move freely into the vertical open ended outflow tube during antrum contractions The effect of distension may have more than one explanation It could simply be that this brings the test agent into contact with a larger area of antral mucosa On the other hand it may facilitate the penetration to the gastrin producing and storing cells These cells are still unidentified but have been suggested to be modified ganglion cells of Meissner s plexus (Baugh *et al* 1958 Woodward *et al* 1963) receptor cells located among the columnar epithelium and connected with the gastrinproducing cells above the muscularis mucosae providing a local reflex for release of gastrin (De La Rosa Woodward and Dragstedt 1965) or endocrine like cells in the body of the pyloric glands (Solcia Vassallo and Sampietro 1967) Gastrin activity of antral mucosa and submucosa has been determined in sections of vertical mucosal levels in dogs and cats (Broome Fyro and Olbe in press) The highest activities occurred in the deeper parts of the mucosa proper, indicating the probable localization of the gastrin producing cells within the antrum glands

As can be seen from Fig 3 maximal acid output from the fundic pouch occurred

within the first hour of instillation of a fixed amount of ethanol and was followed by a rapid fall This is explained by rapid disappearance of ethanol from the antrum, probably by absorption (Elwin, to be published)

Concentrations of ethanol used to stimulate acid secretion at oral ingestion or by direct application in the stomach vary In the test meal in practice generally a 7 % alcohol solution is introduced into the previously emptied stomach (Newman and Mehrtens 1932) Experimental use of ethanol as a tool to study mechanisms of gastrin release and inhibition includes 5 % (Kreuger and Mac Intosh 1937, Woodward and Dragstedt 1960, Maitrya 1963), 7 % (Irvine, Watkin and Williams 1960) and 10 % concentrations (Woodward *et al* 1957, Nyhus *et al* 1960 Shapira and State 1961) Woodward *et al* (1957) could show that 10 % alcohol caused about 50 % stronger response than 5 % However, so far no direct relationship between the administered amount of this chemical stimulus and release of gastrin has been demonstrated In the present study a dose response related release of gastrin with ethanol was found in 4/6 dogs Continuous antral perfusion with alcohol at a constant rate causes a peak level of acid output from a Heidenhain pouch within 1 to 2 hrs followed by a drop below this level within the next 3—4 hrs (Shapira, Morgenstern and State 1960) Sometimes this unexplained fall in secretory response can be very pronounced To avoid interference of this phenomenon the experimental procedure, which is described in the section on results, was chosen to create the dose-response curves The experimental findings were very similar for Heidenhain and Pavlov pouch dogs but Pavlov pouch dogs showed a more pronounced acid output at the maximal stimulatory ethanol concentration. 4 % ethanol (v/v) seems to be the threshold concentration for release of antral gastrin In the majority of the dogs there was a considerable decrease of acid output following perfusion with 32 % ethanol (v/v) Possible explanations offer a local anestetic effect of strong solutions of ethanol inhibition of release of gastrin by changes of cell permeability, denaturation of proteins etc

Release of gastrin by ethanol is sensitive to changes of pH and reduction of pH to 1 0—1 5 of the administered alcohol solution inhibits the effect (Woodward *et al* 1957, Nyhus *et al* 1960) It has also been reported that already acidification of an ethanol solution to pH 3 will reduce acid output from a denervated gastric pouch significantly (Irvine *et al* 1960) The latter supposition is not in accordance with the findings in this investigation (Fig 6) In studies on gastrin release by ethanol antrum pH must be controlled regularly and buffered solutions should be used to keep the acidity at a constant pH This is particularly true if some acid secreting mucosa happens to be left in the isolated antrum pouch Administration of an ethanol solution in such cases would cause not only release of gastrin but following this a secondary stimulation of the oxyntic glands left in the antrum pouch which would tend to lower pH even more In the investigation discussed above no measurements of actual antral pH were reported The slope of the pH curve (Fig 6) for gastrin release by ethanol is independent of the ethanol concentration (Table V) pH curves for gastrin release by 5 % 10 % and 16 % ethanol show the same general feature The ethanol molecule remains unchanged with variations of pH Total amount of gastrin released

at a certain pH will depend primarily on ethanol concentration and administered amount

It is difficult to know if there still is release of gastrin with ethanol solutions of high acidity This must be further elucidated However, figures derived from this investigation coincide with findings of others (Daves *et al* 1965), showing that acidification of a 10 % alcohol solution to pH 1 5 causes 81 % inhibition of acid output

The pH curve for gastrin release with alcohol is almost identical with the pH curve for gastrin release during vagal stimulation (Olbe 1964)

Release of gastrin by ethanol is further influenced by some cations High concentrations of K and Ca^{++} inhibit the effect whereas high concentrations of Na^{+} and Mg do not The mechanism is not known but alcohol has recently been shown to exert pronounced effects on conductance of axon membranes, cell permeability etc (*e g* Moore Ulbricht and Takata 1964)

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1962 1963, Kirshner 1962a and b) This mechanism is blocked by reserpine so that exogenous NA cannot accumulate in the adrenergic nerves after reserpine treatment due to intraneuronal deamination by monoamine oxidase (MAO) However when the effect of MAO also is eliminated after MAO blockade NA is accumulated within the nerves and then the membrane pump mechanism can be studied separately from the ATP Mg^{++}-dependent incorporation of NA into the amine granules (see Malmfors 1965 Hamberger 1967)

The aim of the present investigation is to study the effect of nerve activity on the intraneuronal NA accumulation by the membrane pump mechanism

Material and methods

Adult female albino rats [illegible] 200 [illegible] Under pentobarbital anesthesia [illegible] all the animals by electr[illegible] voltage of 4—8 which w[illegible] [illegible] amine (Regitine®, 5 mg/kg i p [illegible] before the stimulation NA (0 1 mg/kg) was infused via one of the lingual veins during the first 10 min of the stimulation.

The animals were killed by decapitation immediately after the sympathetic stimulation The eyes were removed and the irises were prepard as whole mounts dried treated with formaldehyde gas of optimum humidity at +80° C for 1 hr and examined in a fluorescence microscope (for details see Malmfors 1965) according to the fluorescence method of Falck and Hillarp (see review by Corrodi and Jonsson 1967)

The submandibular and sublingual salivary glands were removed together homogenized and extracted in 0 4N perchloric acid The NA was determined by chromatography and photofluorometry according to Häggendal (1963)

Results

The specific fluorescence formed at formaldehyde gas treatment of NA was absent at 4 or 16 hrs after reserpine pretreatment It could however be restituted with exogenous NA after pretreatment with a MAO inhibitor The appearance and intraneuronal distribution of the restituted fluorescence was different from the normal ones In normal animals the nerve terminals showed a varicose appearance while in animals pretreated with reserpine and a MAO inhibitor the terminals showed a smooth appearance after the restitution (see Malmfors 1965) In experiments with the same or other organs determinations of the NA levels have shown NA accumulation in reserpine treated animals provided that MAO had been inhibited (*e g* Anden, Carlsson and Waldeck 1963)

When the intensity of the fluorescence in the adrenergic nerves of the irises on the two sides of the same animal was compared it was *in every animal found to be* lower on the stimulated side than on the unstimulated one This difference appeared to be more prominent in animals which had not been treated with phentolamine and which had been stimulated at 20 imp/sec

The results from the biochemical estimations (Fig 1) show that the NA levels in all the groups were lower on the stimulated side than on the unstimulated one

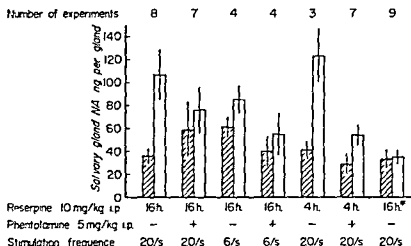


Fig 1 The noradrenaline (NA) content (ng per gland) in the rat salivary glands after nerve stimulation 6 or 20 imp/sec during infusion of NA (0 1 mg/kg i v) after pretreatment with reserpine (10 mg/kg 4 or 1 hrs before the stimulation) and malamide (100 mg/kg 2 hrs before the stimulation) In some cases al o phentolamine (5 mg/kg) was given The empty columns represent the unstimulated side and the striped ones represent the stimulated s de

* These results were obtained when the stimulation (20 imp sc was performed 15 min *after* administration of NA 0 1 mg/kg i v) to rat 4 pretreated with re erp ne (10 mg/kg) and malamide 100 mg/kg 16 hrs and 1 hrs before the stimulation respectively (Haggendal and Malmfors Acta physiol scand 1968 to be publ)

($p<0.001$ for the total material analysis according to the difference method) The difference appeared to be more pronounced after 20 imp/sec than after 6 imp/sec Pretreatment also with phentolamine seemed to somewhat lower the NA levels on both sides but still there was a significant difference between the two sides $p<0.005$ for the three groups taken together Principally the same findings were obtained when the stimulation took part 4 hrs as 16 hrs after reserpine treatment, the corresponding NA levels being about the same at both intervals

Discussion

From the present results it is obvious that nerve stimulation diminished the accumulation of NA in the adrenergic nerves of the iris and in the salivary glands There seems to be several mechanisms which may explain the results

First, a decreased blood flow to the stimulated tissues could have reduced the amount of NA available for uptake due to a vasoconstrictor effect of the stimulation. However following the reserpine pretreatment any physiological responses of the nerve stimulation were not found even when exogenous NA had been taken up after MAO inhibition Haggendal and Malmfors 1968) This will be more discussed in the following section. Furthermore after blockade of any remaining vasoconstrictor effect with phentolamine there was still a significant difference between the stimulated and unstimulated side though this difference appeared to be

reduced after phentolamine The accumulation of NA on both sides appeared further to be somewhat decreased after phentolamine This might be due to some degree of changed circulation or perhaps to an effect of phentolamine on the uptake by the membrane pump

Second, the exogenous NA taken up may have been released by the stimulation. This has been suggested by Trendelenburg (1966) as an explanation to the supersensitivity to NA induced by continuous stimulation in animals pretreated with reserpine and a MAO inhibitor However, in another series of experiments of ours when stimulation was performed after that NA had been administered i v to reserpine nialamide pretreated animals there was no or very little effect of stimulation on the levels of accumulated NA Furthermore, no functional responses were observed during the stimulation (Haggendal and Malmfors 1968) These results thus did not indicate any release of NA under such conditions However, the possibility that NA is released in immediate connection with the uptake of exogenous NA cannot be excluded If this is the case there might also be circulatory effects on the uptake at the stimulation

Third, the decreased NA amounts on the stimulated side may be due to an intermittent inhibition of the membrane pump mechanism caused by the nerve impulses This might also be suggested as an explanation to the results of the above mentioned experiments of Trendelenburg (1966) The results appear to indicate a stronger inhibition at 20 imp/sec than at 6 imp/sec suggesting an increasing blocking effect on the membrane pump at increased nerve impulse frequencies However it may be observed that when this often used frequency of 20 imp/sec at preganglionic stimulation (which seems to be supraphysiological) is continued for several minutes a gradual reduction of the transmitter quantum released per stimulus seems to take place, which may lead to failure of ganglionic transmission once subthreshold amounts of transmitter are reached (see Folkow, Haggendal and Lisander 1967) This makes it likely that during the time period of 20 min of stimulation at 20 imp/sec there can have been some reduction of the number of impulses that have been transmitted The number of transmitted impulses have in all likelyhood however, as a mean been higher than 6 imp/sec during the time period of 20 min

An uptake inhibition may result from the membrane depolarization caused by the nerve impulse since a very strong inhibition of the membrane pump has been shown *in vitro* after chemical depolarization when Na^+ is exchanged for K^+ (Hamberger and Malmfors 1967) This appears to be in agreement with the present finding that uptake inhibition was more pronounced at higher frequencies of discharge

The results obtained 4 and 16 hrs after reserpine treatment make it likely that the effect of reserpine is largely the same during this time interval with respect to the studied phenomena As no new formed amine storage granules appear to reach the terminals before about 16 hrs (Dahlström 1966 Dahlström and Haggendal 1966), this indicates that the amine storage granules affected by reserpine did not markedly change their behaviour during the present study

An intermittent membrane pump blockade of the discussed type would mean that

the NA released by nerve impulses is not immediately eliminated by reuptake into the nerve terminals but may have time to reach the receptor before membrane repolarization restitutes the membrane pump mechanisms

The skilful technical assistance of Mrs Hjordis Koszeghy and Miss Agneta Wilen is gratefully acknowledged The investigation was supported by grants from the Swedish Medical Research Council (B68 14X 166 04 and B68 12X 711 03) and Magn Bergvalls Stiftelse

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The Effect of Nerve Stimulation on Catecholamines Taken up in Adrenergic Nerves after Reserpine Pretreatment

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Abstract

HAGGENDAL, J and T MALMFORS *The effect of nerve stimulation on catecholamines taken up in adrenergic nerves after reserpine pretreatment* Acta physiol scand 1969 75 33—38

The effect of sympathetic nerve stimulation on the catecholamines (noradrenaline and dopamine) taken up and accumulated after i v administration was studied in the adrenergic nerves of rat iris with the fluorescence method and in the salivary glands with biochemical estimations after treatment with reserpine and a monoamine oxidase inhibitor After administration of noradrenaline (0 1 mg/kg) the stimulation did neither cause a functional response in form of increased pupil diameter or bulb protrusion on the stimulation side nor any decrease in the amount of noradrenaline accumulated in the examined organs on the same side However after administration of dopamine (1 0 mg/kg) there were clearcut functional responses on the stimulated side and a marked decrease of the levels of noradrenaline formed from dopamine These results imply that exogenous noradrenaline cannot enter the proper sites for release by nerve impulses 16 hrs after reserpine treatment but that noradrenaline formed from exogenous dopamine in the doses used and after inhibition of monoamine oxidase can be released by nerve impulses in spite of reserpine pretreatment

In isolated amine storage granules from the adrenal medulla an ATP Mg dependent uptake mechanism for catecholamines has been shown (Carlsson Hillarp and Waldeck 1962 1963 Kirshner 1962a b) Evidence for a similar mechanism has been provided by Euler and Lishajko (1963) for isolated subcellular particles from sympathetic nerves This uptake mechanism is blocked by reserpine (see Carlsson 1965) Reserpine causes a great reduction of the noradrenaline (NA) within the adrenergic nerves and the adrenergic transmission is also seriously affected (Carlsson *et al* 1957) However in spite of reserpine pretreatment the adrenergic nerves can take up and accumulate NA the latter provided the action of monoamine oxidase (MAO) is inhibited (see Malmfors 1965) This reserpine resistent mechanism seems to be located to the cell membrane Furthermore later histochemical experiments indicate that some of the NA taken up may be bound to the amine storage granules (Hamberger and Malmfors 1967)

Evidence for such a reserpine resistent binding of amines to subcellular particles has also been obtained using preparations both from the adrenal medulla and the adrenergically innervated heart (Lundborg 1966, Lundborg and Waldeck 1966, Carlsson *et al.* 1967, Lundborg and Stitzel 1967). Thus, there are at least two different mechanisms in the amine storage granules dealing with the amines: the ATP-Mg^{++}-dependent mechanism which is sensitive to reserpine and the reserpine resistent mechanism. It seems as the two mechanisms are quantitatively utilized in different ways by NA and some NA analogues. Thus, metaraminol uses the reserpine-resistent mechanisms more than α-methyl-NA and NA, while NA uses the ATP-Mg-dependent mechanism more than α-methyl-NA and metaraminol (Lundborg and Stitzel 1967). Dopamine (DA) seems to use both mechanisms (Lundborg 1966).

DA-β-hydroxylase, which transforms DA to NA, is attached to the amine storage granules (Kirshner 1962b, Potter and Axelrod 1963). *In vitro* studies have shown that reserpine interferes with the β-hydroxylation of DA (Kirshner 1962, Rutledge and Weiner 1967) by preventing DA to reach the enzyme. Using α-methyl-m-tyramine and α-methyl-DA, Meish, Carlsson and Waldeck (1967) showed *in vivo* that after reserpine these compounds could reach the DA-β-hydroxylase. However, as α-methylated amines seem to be handled by the amine storage granules somewhat different than their non-methylated analogues, DA and NA have been used in these studies.

The aim of the present work thus is to obtain information about the catecholamines present after NA and DA injections and to study the effect of nerve impulses on the accumulated amines.

Material and methods

Adult female albino rats (Sprague Dawley) weighing approximately 200 g were used. The sympathetic nerves to one of the irises were stimulated in all the animals by electric stimulation of the preganglionic trunk under pentobarbital anesthesia (20 mg/kg i.p.) (see Malmfors 1965). Twenty biphasic impulses were given per second during 20 min and the voltage was 5 to 8 which was found to give a supramaximal stimulation as judged by the degree of dilatation of the pupil and the protrusion of the eye ball in normal animals. The diameter of the pupil was measured with a scale in the eye piece of the dissection microscope. All the animals were given reserpine (100 mg/kg i.p.) 16 hrs before the stimulation. Some of the animals received nialamide (100 mg/kg i.p.) 2 hrs before the stimulation. NA (0.1 mg/kg i.v.) or DA (1 mg/kg i.v.) were given 15 min before the stimulation, which lasted 20 min. The animals were killed by decapitation immediately after the stimulation. The eyes were removed and the irises were prepared as whole mounts, dried, treated with formaldehyde gas of optimum humidity at 80° C for 1 hr and examined in a fluorescence microscope (for details see Malmfors 1965) according to the fluorescence method of Falck and Hillarp (see review by Corrodi and Jonsson 1967). The salivary gland (the submandibular and the sublingual glands) were removed together, homogenized and extracted in 0.4 N perchloric acid. The DA and NA were determined by chromatography and photofluorometry according to Carlsson and Waldeck (1958) and Häggendal (1963), respectively.

Results

In the animals not pretreated with nialamide there were after DA or NA administration no clear signs of catecholamines in the examined organs, iris and salivary glands, neither on the stimulated side nor on the unstimulated side. There was no effect on the diameter of the pupil during the stimulation in these animals.

TABLE I The effect on pupil diameter bulb protrusion fluorescence intensity in the adrenergic nerves of iris and catecholamine content in salivary glands (submandibular and sublingual glands) by preganglionic stimulation (20 imp sec during 20 min) of the right sympathetic trunk in rats pretreated with reserpine (10 mg/kg i p) 16 hrs earlier nialamide (100 mg/kg i p) 2 hrs earlier and noradrenaline (0 1 mg/kg i v) or dopamine (1 mg/kg i v) 15 min before the stimulation Symbols for the average fluorescence of the adrenergic ground plexus in iris + + + = strong + = weak Number of estimations = n Mean values ± s e m are given t test according to the difference method

Catecholamine administered	Pupil diameter and bulb protrusion	Fluorescence intensity right iris	Fluorescence intensity left iris	Catecholamine content per salivary gland: noradrenaline right in ng	noradrenaline left in ng	dopamine right in ng	dopamine left in ng
noradrenaline	unchanged	+ + +	+ + +	33 ± 7 8	35 ± 5 9		
				n	9		
				p >	0 5		
dopamine	increased	+	+ +	5 ± 0 9	14 ± 2 9	33 ± 17	112 ± 68
				n	12	n =	6
				p	0 01	p	0 10

The results from the animals pretreated with nialamide are summarized in Table I

After administration of NA there was a restitution of the specific fluorescence in the adrenergic nerves of the irises and an accumulation of NA in the salivary glands The fluorescent nerves showed the smooth appearance earlier described after this treatment However there was no significant difference between the stimulated side and the unstimulated side neither in the fluorescence intensity nor in the NA content Furthermore there was no effect by the stimulation on the pupil diameter or the protrusion of the eye ball

After administration of DA there was a restitution of fluorescent fibres on the unstimulated side with the varicose appearance earlier described after DA given to an animal pretreated with reserpine and nialamide (Malmfors 1965 However on the stimulated side there was a marked reduction and change of the specific fluorescence The fluorescence of the non terminal axons were unchanged or even more pronounced compared to the unstimulated side but in the terminals especially the fluorescence of the varicosities was diminished leaving only very weakly fluorescent and smooth terminals left

It was possible to detect NA as well as DA in the salivary glands on both sides after administration of DA to the animals pretreated with reserpine and nialamide In all cases there was a marked reduction in the NA content and the DA was reduced in 5 animals of 6 on the stimulated side as compared to the unstimulated side

During the stimulation there was a clearcut increase of the pupil diameter and the bulb protrusion

Discussion

From the results it is obvious that exogenous NA can be taken up by the membrane pump and accumulated in the salivary glands as well as in the adrenergic nerves or iris after reserpine treatment provided that the effect of MAO is inhibited as earlier has been shown (e g Andén Carlsson and Waldeck 1963 Malmfors 1965) This accumulated NA is probably located extragranularly in the whole adrenergic neuron (Hamberger *et al* 1964 Malmfors 1967) However small amounts appear to be attached to the granules in spite of reserpine treatment (Carlsson *et al* 1967 Hamberger and Malmfors 1967 Lundborg and Stitzel 1967 van Orden Benesch and Giarman 1967)

If DA is given to a reserpine pretreated animal both NA and DA are accumulated after MAO inhibition and probably both the amines are responsible for the fluorescence found in the adrenergic nerves This implies that DA can be converted to NA *in vivo* in spite of reserpine pretreatment Kirshner (1962b) claimed that reserpine *in vitro* interferes with the uptake of DA in a particulate fraction of the adrenal medulla but does not inhibit the formation of NA once the DA has been taken up Using α methyl m tyramine and α methyl DA Meish Carlsson and Waldeck (1967) found that β hydroxylation occurred despite the blockade of the storage mechanism by reserpine Rutledge and Weiner (1967) demonstrated in isolated rabbit atria incubated with labelled DA a dose dependent reduction of the formation of labelled NA after reserpine Since our present results are obtained after MAO inhibition and furthermore DA compared to NA is a better substrate for MAO (Weiner 1960 Jonason and Rutledge 1968) the DA concentration may be relatively high extragranularly The possibilities for DA to enter the granules may thus in the present studies be enhanced utilizing a reserpine resistent uptake mechanism compared to normal conditions

Reserpine has been shown to decrease the NA content in tissues and to interfere with the adrenergic transmission in all probability due to functional lack of the transmitter Refillment of the catecholamine stores especially in the brain with L-DOPA which restores the function supports this idea (for review see Carlsson 1965) After repeated doses of reserpine it is shown that the normal function cannot be correlated to the absolute levels of monoamines but to changes of a small fraction being less than 10 per cent of the normal contents (Häggendal and Lindqvist 1964a, b) After a single dose of reserpine normal functional responses at nerve stimulation returns after 36—48 hrs which in time is correlated to the ability of the tissues to retain labelled NA while the NA levels in the tissue still are very low (Andén Magnusson and Waldeck 1963 Andén and Henning 1966)

After 'refillment' of the transmitter with exogenous NA 16 hrs after reserpine and 2 hrs after nialamide treatment no release of NA or any functional responses could be observed The amounts of NA found in the salivary glands were about 15—20 per cent of the normal NA content Even if some of this NA is extraneuronally bound most of the NA probably is located within the nerves and some of it within the varicosities However the lack of functional responses after stimulation in the

present investigation strongly suggests that reserpine after MAO inhibition prevents the exogenous NA to enter the 'proper sites' from which it can be release at nerve activity

After administration of DA, however, the functional responses, the presence of NA in the tissue and the decreased NA levels on the stimulated side, suggest that DA has passed into the granula, where it has been β-hydroxylated to NA and that this new formed NA has been released at the nerve activity Even DA may have been released since in 5 of 6 cases also the DA levels were lower on the stimulated side However, the receptor stimulating effect of DA is very small compared to the NA In all probability the NA has been released by the nerve impulses from the 'proper site of release'

From the results it thus seems as if NA formed from exogenous DA can be released after reserpine treatment and MAO inhibition The NA has to be formed in connection to the granules where the β-hydroxylase is located The entrance of this NA to "the proper site for release" or 'the functional pool' is obviously not inhibited by reserpine after MAO inhibition, while the exogenous NA does not appears to be able to reach this site

The difference in the results received after exogenous NA and exogenous DA administration may be expressed as a betted ability for DA to utilize the "reserpine resistent uptake mechanism" into the granules under the present conditions It may, however be observed that the dose of NA used was 10 times less than the dose of DA

The restitution of the function was in the present investigation studied 16 hrs after reserpine However, also 4 hrs after reserpine the same results have been obtained This time apepars definitely to be too short for any fresh granules to be formed in the cell bodies and transported down to the tissue (Dahlstrom 1966 Dahlstrom and Haggendal 1966) The restitution of the function observed after DA thus seems to occur in connection with granules affected by reserpine It must however be observed that the restitution occurs after MAO inhibition and the mechanism may not be compared to the normal restitution after reserpine, which occurs much later

The skillful technical assistance of Mrs Hjordis Koszeghy and Miss Agneta Wilen is gratefully acknowledged The investigation was supported by grants from the Swedish Medical Research Council (B68 14X 166 04 and B68 12X 711 03 and Magn Bergvalls Stiftelse

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Inhibitory Processes in Gustation

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Abstract

HELLEKANT, G *Inhibitory processes in gustation* Acta physiol scand 1969 *75.* 39—48.

A stage of decreased excitability of the gustatory receptors after taste stimulation was demonstrated in a previous study (Hellekant 1968) This conclusion was based on the observation that when the same stimulus was applied two or more times to the tongue, the first exposure depressed subsequent responses, although the tongue was continuously rinsed with water between the periods of stimulation An attempt was made to explain this observation by applying the idea of Beidler's general taste theory According to his theory the gustatory response is related to the number of receptor sites which bind stimulus when it is applied In the above mentioned study it was suggested that the first stimulus bound to the sites and that the disengagement after stimulation took time during which fewer sites were available to the second stimulus This was seen as a diminution of the gustatory response to the second stimulation If it is assumed, in agreement with the idea of Beidler (1962, 1965, 1967), that there are given receptor sites for each taste substance, it may be concluded that the depression should be less pronounced if two stimuli, which do not compete for the same receptor sites, are applied after each other The more the stimuli resemble each other from the receptor's point of view, the more they will compete for the sites and consequently depress the responses of each other Thus the method seems to offer a possibility to estimate the similarity between gustatory stimuli from the receptor's point of view

Methods

solutions The sequence of stimuli is schematically illustrated in Fig 1 The response to the first

Results

It was observed that the first gustatory stimulus usually diminished the response to the second, when they were separated by the short interval used in this series. However, the degree of diminution depended very much on the various pairs of stimuli Table I contains all results obtained when the 13 solutions of this study were combined in the way described under methods The results of any of these combinations can be found in the intersection between the row containing the *first solution, which is listed to the left of the array, and the column which contains* the second solution listed on the top A value of 100 indicates that no effect was observed, a value above 100 indicates that the response to the second solution was enhanced by the first and a value under 100 indicates that the second response was depressed The mean difference between the largest and smallest value of each set of values in Table I is 16 % ± 2 S E A difference less than 20 % was therefore not considered real Values obtained during the same series of experiments are listed in the same row The letters to the right represent the preparations

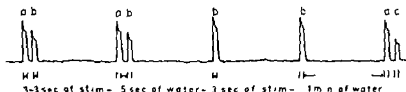


Fig 1 Illustrates schematically the sequence of stimuli and the length of the intervals between them The letters a, b and c represent different stimuli The right pair a—c, indicates the beginning of a new sequence

The rows of Table I were arranged according to the mean value for the depression the solution in the row caused, while the columns were arranged according to the mean values of the depression they exhibited. Thus the solution which on the average exerted the strongest depression is in the top row and the one which showed least depression in the outermost right column. It can be observed from this arrangement that in general K_2SO_4 and Li_2SO_4 caused the strongest depression and that $MgCl_2$ and NaCl were most depressed. Furthermore, Table I shows that no solution was entirely inert in affecting the second response. There is also no substance whose response always was unaffected by being the second stimulus, though the responses of the acids and K_2SO_4 in general were little affected.

It was shown in an earlier study (Hellekant 1968) that the recovery of the gustatory receptors after stimulation was faster in some animals than in others. These characteristics may contribute to the differences between the values of each cell in Table I. The assumption that there is no consistent difference between the values of two preparations to the same set of stimuli was therefore tested with a simple sign test. The assumption was rejected ($\alpha = 0.05$). This indicates that factors which depended on the preparation added to the variation. The observation in the earlier study (Hellekant 1968) that the recovery of gustatory receptors is faster in some preparations than in others was also supported.

It may be suggested that a better base for the comparison between the effects caused by the different combinations of solutions can be reached by ranking the depressions in each series with ordinal numbers. Such a procedure diminishes the effect of individual variations between the preparations and rules out the previous mentioned general difference between the stimuli in the strength of the depressions caused. The values in each row of Table I were ranked and the mean value for each cell calculated. Series with less than 12 values have not been used. The result of this manipulation is presented in Table II. The order of solutions differs in Table II from that of Table I. In Table II the substances were arranged according to their effect on themselves. This was done in such a way that the one which exerted its strongest depression on itself is in the top row. This can be seen by comparing the values along the diagonal from the upper right corner to the lower left. If stimulus similarity is the only factor responsible for the depression these values should be the minima of each row. Table II shows that choline chloride, $NaNO_3$, $MgCl_2$, $CaCl_2$ *and possibly acetic acid (Table I) fit into this category. The differences between the* maximum depression observed and the depression caused by LiCl and Li_2SO_4 on themselves is small as can be seen from Table II. The discrepancy is insignificant and they may therefore be regarded as exerting their strongest effect on themselves.

Erickson, Loetsch and Marshall (1965) expressed as correlation coefficients the degree of similarity of the neural responses produced by two solutions. They recorded from single chorda tympani nerve fibres of the rat, using the number of impulses elicited by the chemicals during the first sec of stimulation as the basis for their calculation. In their study a high correlation coefficient indicates a high similarity between the neural input of two stimuli, *i.e.* the stimuli were probably

TABLE I

1st solution	2nd solution $MgCl_2$	$NaCl$	$LiCl$	$NaNO_3$	Chol Cl
0 3 M Li_2SO_4	18	29	19	14	35
	31	30	20	20	42
0 3-M K_2SO_4	27	19	52	75	39
	29	11	29	36	36
0 01 M QHCl	64	18	57	59	44
	78	35	67	50	55
0 3-M $NaNO_3$	33	29	15	19	41
	43	66	48	35	51
0 3 M LiCl	48	62	53	42	45
	28	35	10	33	39
0 1 M KCl	56	39	41	39	63
	52	50	44	56	60
	45	65	37	33	
0 016-M HCl	32	48	67	87	69
0 05-M NH_4Cl	64	74	71	44	75
	59	40	39	41	52
0 1 M $MgCl_2$	44	55	35	43	44
	46		64		61
	22	60	58	56	57
M $CaCl_2$	64	43	82	83	64
	44	42	37	65	52
0 1 M Cholin Cl	77	57	77	72	49
	69	77	47	51	36
0 3 M NaCl	44	82	40	54	79
	49	63	79	81	63
	96	59	44	34	50
	56	77	59	41	
0 05 M HAc	78	109	88	76	87
	80	73	107	133	84

similar from the receptor s point of view In the present study such a similarity should be seen as a strong interaction between two stimuli A comparison between the values of Table II and those by Erickson *et al* may therefore be of interest Values of the seven first rows of Table II with the exception of those of $CaCl_2$ were used The reason for this limitation will be discussed later The correlation between the results of the two studies was -0 5 Using one tailed test of significance, such a correlation was likely to occur by chance less than once in hundred times It can therefore be concluded that the relationship between stimuli found by Erickson *et al* and some of those in this study agree with each other despite the fact that the results were obtained from two different species rat and cat

QHCl	$CaCl_2$	KCl	Li_2SO_4	NH_4Cl	HAc	HCl	K_2SO_4	
61	24	43	11	60	100	93	100	C
58	29	55	34	65	92	82	92	G
51	36	34		62	98	93	75	E
92	23	29	38	68	98	94	56	G
47	93	29	59	50	62	88	47	C
38	86	47	64	60	98	102	88	G
59	53	79	43	79	97	90	101	B
39	40	77	70	95	98	95	117	E
58	68	88	77	78	95	93	107	B
58	49	84	46	75	100	99	112	C
55	63	56	33	79	86	93	106	C
72	49	62	77	81	108	100	92	E
	64	67						F
29	76	68	102	73	78	87	85	D
81	77	67	82	86	109	98	90	A
71	48	50	24	72	92	95	100	C
65	67	71	108	64	83	103	103	B
57	68	92	100	93	89	93	119	D
68	63	78	105	96	100	119	100	E
50	48	78	118	104	101	94	103	B
58	33	52	63	104	94	100	107	D
72	90	107	92	100	95	95	117	C
84	92	102	117	101	94	100	104	G
88	82	79	81	105	111	103	110	A
63	81	68	80	115	106	98	104	B
64	[illegible]	74	50	109	100	101	118	D
								F
71	89	100	96	49		8	93	A
49	90	70	75	81		100	85	B
					49	66		F

The remaining six substances did not exert their maximum effect on themselves, *especially not* K_2SO_4, NH_4Cl *and HCl*. They exerted a much stronger effect on most other substances. This will be discussed below.

It was also demonstrated in the earlier study of this series (Hellekant 1968) that different chemicals showed different rates of recovery. An attempt was made in that study to express the rate of recovery to each solution by the use of a time constant. This was simply defined as the time in sec when 2/3 of maximum response was reached during the recovery. In this study it may be surmised that such a factor may be of importance because some substances will bind to the receptor sites longer than others and thereby affect the response of the second solution for a longer ti

TABLE II displays the mean values obtained after ranking the values of each row in Table I

	Chol Cl	$NaNO_3$	$CaCl_2$	$MgCl_2$	HAc
Chol n.Cl	1 0	3 3	7 0	4 8	8 8
$NaNO_3$	5 5	1 5	5 0	4 0	12 0
$CaCl_2$	5 3	8 5	1.5	3 5	10 0
$MgCl_2$	3 3	2 0	6 3	1 8	8 8
HAc	6 3	7 5	8 5	4 5	—
LiCl	3 5	2 0	7 0	2 5	12 0
Li_2SO_4	0	1 8*	4 0	4 0	12.5
QHCl	4 0	6 3	11 5	10 0	11 0
NaCl	3 3	4 1	8 0	5 3	11 7
KCl	7 3	3 8	5 3	5 3	12 0
K_2SO_4	5 8	8 0	3 0*	3 0*	12 5
NH_4Cl	6 5	2 5	6 0	5 0	12 0
HCl	6 0	11 5	8 0	2 0	9 0

may explain the difference in the depressions observed when the order between two solutions was reversed The assumption was tested with a sign test for the data of Table II It could be concluded for the results of the test that this factor may be responsible for the differences observed when the order between quinine and the other substances was reversed but not for the other stimuli

Discussion

The result of this study can be summarized in three expressions if an arrow pointing downwards ↓ represents "depress and the letters a b represent both stimulus and the "response to stimulus This will give us the three expressions

1 a ↓ a > a ↓ b
2 a ↓ b < b ↓ a
3 a ↓ b > b ↓ b

The following discussion is an attempt to draw conclusions from these three cases and to present tentative explanations for them

a ↓ a > a ↓ b

— — — —

The comparison with the results of Erickson *et al* 1965) was restricted to the data of six of the first seven substances in Table II because they exerted their strongest effect on their own second response This behaviour was predicted by the hypothesis on which this study was based Calcium was then excluded from the comparison because its effect was not regarded as caused exclusively by its competition with other stimuli for the receptor sites It was felt in the light of the numerous observations on the general effect of calcium on membrane that it also exerted what from this study's points of view may be called nongustatory effects It was for example observed several times that calcium ions could restore the sensitivity of gustatory receptors

LiCl	Li_2SO_4	QHCl	NaCl	KCl	K_2SO_4	NH_4Cl	HCl
3.8	10.5	4.8	3.5	11.5	12.5	10.5	9.3
3.0	7.0	5.0	5.0	9.8	13.0	10.0	10.8
4.5	10.5	5.5	2.0	5.8	12.0	12.0	10.0
3.0	11.7	5.3	5.0	8.0	11.0	7.8	11.0
9.0	7.0	1.5	7.5	6.5	8.5	3.5	7.8
2.5	7.0	6.5	5.0	10.0	13.0	9.0	11.0
2.8*	3.5	9.5	5.0	8.0	12.5	9.5	11.0
7.5	7.8	3.3	1.0*	2.5*	7.8	6.0	12.5
3.0	5.5	5.7	4.8	5.2	12.0	12.0	10.3
2.5	5.0	6.5	2.8*	6.8	12.0	10.0	12.0
5.5	8.0	8.5	1.0*	3.5	9.3	9.0*	11.5
3.0	5.0	8.5	4.0*	4.5	12.0	10.0	12.0
4.0	13.0	1.0*	3.0*	5.0	10.0	7.0*	11.5

which had been damaged by acid Furthermore the suppression of the recorded baseline after sodium chloride exposure which was described by Pfaffmann and Powers (1964) for the rat was also observed in this study for the cat This suppression could be avoided for a considerable time if the tongue was rinsed with calcium chloride

The remaining six substances were not included in the comparison because their effects on other substances were greater than their effects on themselves Thus competition for the receptor sites by similar substances could not explain the results without invoking an additional factor

a ↓ b ≠ b ↓ a

Table I reveals that changing the order between two stimuli in several cases caused a considerable difference of the depression measured This observation may be explained by assuming that the receptor fields of different substances overlap and differ in size That is the sites of one kind of stimulus are more numerous than those of another This has also been suggested by Beidler 1962 1967

Fig 2 emphasizes the consequence of such an assumption It shows schematically *two receptor fields A and B which bind stimulus a and b respectively The fields* overlap, *i e* A is to some extent also stimulated by B and *vice versa* The fields have different sizes *i e* the sites which mainly combine with b are more numerous

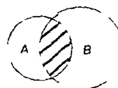


Fig 2 Shows schematically two populations of receptor sites of different sizes They combine to some extent with each other s stimulus It is implicit that the joint area constitutes a larger part of A then of B

It is evident that the response to a will be more affected than that to b when the order between the stimuli is reversed. This idea can be applied for the data in Table I. Conclusions concerning the relative number of receptor sites to two stimuli may be drawn by comparing the values for the depressions obtained, when the order between the stimuli was reversed

$$\underset{\sim}{a} \downarrow \underset{\sim}{b} > \underset{\sim}{b} \downarrow \underset{\sim}{b}$$

This series of experiments revealed also that in some cases stimulus a depressed b more than b depressed its own second response. These observations are pronounced and consistent as can be seen by comparing the individual data of Table I. They can hardly be regarded as artifacts. The asterisks of Table II mark the stimuli of interest in this respect.

It is felt that two tentative explanations should be considered for this observation. The first one is based on the idea of Beidler (1962). He suggested that the binding of a substance to the receptor sites does not necessarily imply that it elicits a response. Observations obtained by several authors (*cf* Oakley 1966) indicate that one constituent of a mixture of gustatory stimuli can directly interact with the receptor sites of the other and inhibit the response to the other. A similar mechanism for the stimuli of Table II would then imply that stimulus a binds for a longer period to and stimulates less the receptor sites of b than stimulus b itself does.

The other tentative explanation is based on the observations that in general most taste fibres respond to a range of taste stimuli, more to some and less or not at all to others (*Pfaffmann 1941, 1955, Cohen, Hagiwara and Zotterman 1955,* Liljestrand and Zotterman 1956, Nagaki, Yamashita and Sato 1964, Hellekant 1965, Bartoshuk 1965).

There is therefore support for the idea suggested by Erickson (1963, 1967) and Erickson *et al* (1965) that the sensitivity of the gustatory neurons displays a Gaussian like function (NRF) when their frequency of response is plotted versus an arbitrary but fixed order of stimuli, a stimulus dimension. The advantage of such an arrangement, quite beside its relevance, is that it will considerably diminish the number of fibre types necessary to mediate the almost infinite number of gustatory stimuli, as the same neuron can respond to several stimuli. The perception does not depend on the absence or presence of a response in a neuron but on the relation between the responses in a responding set of neurons. Neither is it connected with some kind of temporal pattern which implies that there is no relation per se between time and sensation.

However, a wide range of sensitivity of each neuron increases the difficulties for CNS to identify the sensation in question, as the differences between the activity from the neurons close to each other along the "stimulus dimension" will diminish. It can therefore be concluded that a high degree of specificity, *i.e.* each neuron responds only to few stimuli, will demand more types of neurons, while a low degree, *i.e.* many kinds of gustatory stimuli elicit a response, will make the identification of a stimulus more difficult.

It is conceivable that a mechanism which minimizes this limitation by enhancing the difference in the responses between the neurons would be of value This can be accomplished by suppressing the activity of neurons, which because they have a similar 'spectrum" of sensitivity also respond This will then accentuate the difference between two similar tasting substances Such a suppression can be accomplished through a slight change of the model by Erickson (1963) His NRF would then display negative i e inhibitory phases laterally As for the data of this study, such a mechanism can be suspected for the observations of the alkali salts of Table II, while the first tentative explanation may apply for *e g* HCl

There is morphological support for the assumption that the gustatory message can be modified All electronmicroscopical studies (*e g* De Lorenzo 1963) demonstrate a nerve plexus at and below the taste cells This may indicate same data processing at the periphery according to Beidler (1965) Two types of gustatory nerve fibres have been suggested of which one may be efferent since it contains postsynaptic vesicles (De Lorenzo 1963 Nemetschek Gansler and Ferner 1965) Electrical connections between some of the papillae containing gustatory receptors but not between others have also been demonstrated by Rapuzzi and Casella (1965) for the frog However, reciprocal inhibition between the neural elements has not been *demonstrated though the existence of other efferent influences has been supported* (*cf* Halpern 1967) But it is felt that the results of this study indicate that this possibility might also be considered *

Finally the results and the discussion of this study can be summarized as follows 1 The solution depressed its own response more than the response of any other solution This may be interpreted as being caused by a competition between the stimuli for the receptor sites The observed depression was regarded as a measure of the similarity between two substances 2 The degree of depression changed when the order between two stimuli was reversed Overlapping and differences in the number of receptor sites were suggested as tentative explanations 3 The solution depressed the response of another solution more than it depressed its own second response The results indicate that the existence of reciprocal inhibition might be considered in gustation

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* Since this study was written it has been shown at the III Symposium on Olfaction and Taste that the response in a single chorda tympani nerve fibre of the rat during stimulation of one taste bud can be strongly affected by chemical stimulation of surrounding taste buds (Miller pers com 1968)

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Relation of Afferent Nerve Excitability to Impulse Generation in the Frog Spindle

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Abstract

Ottoson D and G M Shepherd *Relation of afferent nerve excitability to impulse generation in the frog spindle* Acta physiol scand 1969 75 49—63

The excitability properties of the sensory nerve and its finer branches in muscle spindle have been studied in isolated preparations using electrical stimulation of the nerve in conjunction with controlled extension of the spindle Characteristic differences in recovery cycle were demonstrated between the trunk fibre and the intracapsular branches The excitability of the afferent nerve was modified by stretch during stretching there was a rise in excitability and a shortening of the recovery cycle in direct relation to the amount of stretch These changes were related to the underlying receptor potential and to local currents in the nerve In the aftermath of stretch the excitability of the nerve as tested with electrical shocks was raised This was in contrast to the depression in mechanical sensitivity as revealed by a test stretch When an extra impulse was elicited by an electrical shock in the course of sustained stretch the succeeding spike train was shifted its general pattern being unchanged It appears that the interjected spike transiently wipes out the currents leading up to the generation of the succeeding spike and resets the impulse generating process. Considerations of the functional properties of the afferent fibre and its branches as found in the present study suggest that the sensory system of the muscle spindle acts in synchrony during stretch

The impulse discharge of the muscle spindle has distinctive patterns during the dynamic and static periods of an applied stretch In earlier studies these patterns have been correlated with the receptor potential (Ottoson and Shepherd 1965 Shepherd and Ottoson 1965) and the mechanical sensitivity of the spindle (Ottoson McReynolds and Shepherd 1968) It was concluded that the impulse discharge was determined by the response in the sensory endings modified by the excitability changes in the nerve and its branches The exact time course and effect of the excitability changes was however impossible to determine by using stretches as both conditioning and test stimuli

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Fig 1 Schematic diagram illustrating arrangement of stimulating and recording electrodes for testing excitability of afferent nerve of isolated spindle (see Methods)

The present paper reports experiments in which the functional characteristics of the sensory fibre and its intracapsular branches were determined by different combinations of paired mechanical and electrical stimuli It will be shown that the excitability cycle of the trunk nerve differs from that of the finer branches By the experimental method used it was also possible to define the excitability changes taking place in the conducting pathways of the sensory system of the spindle during transduction of the mechanical stimulus into the discharge of the nerve

Methods

The experiments were carried out on spindles isolated from the frog s toe muscle *(m ext d g long IV)* The methods for dissection mounting and mechanical stimulation were the same as described in previous papers (Ottoson 1965 Shepherd and Ottoson 1965) The afferent nerve was isolated and dissected clean over a distance of about 1 to 2 mm Electrical stimulation was delivered with chlorided silver-electrodes in connection with an isolation unit (Grass S 4 The recording was done with calomel electrodes connected to a DC-amplifier A typical arrangement of stimulating and recording electrodes is diagrammatically shown in Fig 1 The temperature of the Ringer bath containing the spindle was kept at +18° C

Results

Recovery cycle of the afferent nerve

The excitability of the afferent fibre after the generation of an action potential in the absence of any effects due to stretch was studied by testing the threshold of the nerve with paired electrical shocks A test shock of constant strength was applied at various intervals in the aftermath of an impulse evoked by a conditioning electrical stimulus With a test shock that was just strong enough to evoke an action potential *in the resting nerve the duration of the recovery period could then be determined* Following the refractory period there was a brief period during which the test shock evoked an impulse and finally a prolonged period during which excitability gradually recovered A typical experiment is illustrated in Fig 2 in which the conditioning spike is drawn schematically and the periods during which the nerve responded to the test shock are indicated by closed bars A similar test following a spike elicited by a brief threshold stretch is also included as can be seen the recovery of the fibre was essentially the same in both cases

The exact time course and magnitude of these changes in excitability was found by measuring thresholds at different intervals after a spike These experiments

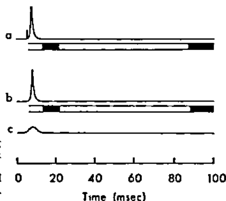


Fig 2 Recovery of excitability of nerve following conditioning spike evoked (a) by electrical shock to nerve, (b) by threshold stretch (c) of spindle Conditioning spike shown schematically, filled bars indicate periods of response to a test shock at threshold strength fo resting nerve

showed that the absolute refractory period lasted until about 2 msec and the relative refractory period until 4 to 5 msec, followed by the interval of heightened excitability mentioned above This increase in excitability was not great, and indeed required some search to be demonstrated It was determined in several preparations by either varying the test shock strength or testing with a given subthreshold shock. Both methods showed that the lowered threshold was a regular phenomenon and constituted a true supernormality (*cf* Graham 1935) A final period of subnormality involved a very slight raising of the spike threshold, lasting up to 60 msec or more A complete cycle from a typical experiment is illustrated by the curve (open circles) in Fig 3 In this case the conditioning stimulus was a minimal threshold stretch

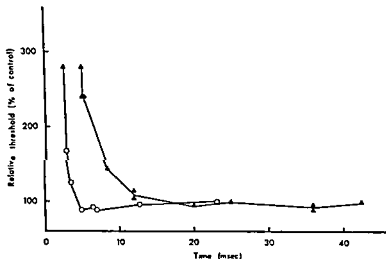


Fig 3 Excitability cycle of afferent nerve Open circles conditioning stretch followed by electrical test shocks Triangles electrical conditioning shock followed by test stretches Conditioning stimuli just above threshold strength

A similar curve was obtained when the conditioning spike was produced by an electrical shock This recovery cycle would be expected for frog peripheral nerve fibres of this diameter (5—10 μ) and at this temperature (about 20° C) (*cf* von Brücke Early and Forbes 1941) As noted in Methods the shocks employed did not exceed about twice threshold in order to avoid damage to the nerve, the end point was on the steep limb of the relative refractory curve (Fig 3) and was considered sufficiently accurate for the purpose of these experiments

When the nerve threshold in the aftermath of a single impulse was measured with a brief stretch applied to the spindle instead of with a shock to the nerve a different recovery cycle was obtained (Fig 3 closed triangles) With this testing situation there was a period of absolute refractoriness of 4 to 5 msec followed by a period of 10 to 20 msec during which the excitability returned in monotonic fashion to its normal level In some cases a longer time was required for complete recovery At these longer intervals the threshold changes were very slight which made it difficult to demonstrate whether or not there was a period of supernormality When the conditioning spike was elicited by a threshold stretch the curve was essentially the same (*cf* Ottoson *et al* 1968 A comparison of the two curves in Fig 3 clearly brings out the different time course of recovery using in one case electrical test shocks and in the other brief test stretches in the same spindle The difference between the curves reveals the characteristic differences in recovery between the trunk nerve and its finer branches as will be further discussed blow (*cf* Fig 12)

ffects of increasing amplitude of stretch on nerve excitability

rief dynamic stretch When stretch was carried beyond the minimum for eliciting a single spike the recovery cycle of the nerve was altered In the simplest case when the amplitude of stretch was increased keeping the duration brief there was a tendency for the periods of absolute and relative refractoriness to shorten For the spindle illustrated in Fig 3 (open circles) a moderately strong stretch caused the absolute refractory period of the nerve to be reduced from 2 6 to 2 2 msec and the recovery curve during the time of relative refractoriness to be correspondingly shifted to the left The subsequent supernormality appeared to be unaffected in magnitude though possibly lengthened in time In some experiments a test shock of given strength was used several other experimental parameters were then varied and their effects on nerve refractoriness compared Table I summarizes the data from a typical experiment and shows that the refractory period shortened with increasing strength and duration of stretch and with increasing strength of testing shock

While these results seemed straightforward a closer analysis revealed effects of stretch which could not be attributed to a simple shift in time course of the recovery cycle of the nerve alone These effects were found by careful testing with very weak electrical shocks Fig 4 illustrates an experiment in which test shocks were introduced following the application of brief stretches of three different amplitudes The testing shock in all runs was of constant strength and about 10 % below the normal spike

TABLE I

Time course of stretch	Refractory period		Decrease in refract period	Strength of elec test rel to thresh
	weakest stretch	strongest stretch		
Brief	4 4 msec	3 2 msec	1 2 msec	below
Brief	4 0	3 1	0 9	above
Sustained	4 0	3 25	0 75	below
Sustained	3 6	2 9	0 7	above

threshold It can be seen that in the control run (with a conditioning spike evoked by an electrical shock) there was but a brief period (at around 6 msec delay) in which the nerve responded to the test shock Since the test shock was below the unconditioned spike threshold this period represented a true supernormality Following a spike evoked by a weak stretch (x 1 threshold) this period was little changed At twice threshold (x 2) stretch, however, the period was definitely changed, beginning earlier and lasting longer (about 4 to 12 msec) As is well known a stronger stretch elicits a greater receptor potential which reaches its peak at the end of the rising phase of stretch and then decays toward baseline with a relatively long time constant The increasing excitability of the nerve with the stronger stretch appears therefore to be related to the higher amplitude of the receptor potential during the early period of decay from its dynamic peak

With still stronger (x 3) stretch there appeared an additional early period of raised excitability This period, coming at the peak of dynamic stretch was not simply an earlier onset of the supernormal period of the nerve (found with 2 x stretch) It could be shown in these cases that if the stretch carried just a bit further a second

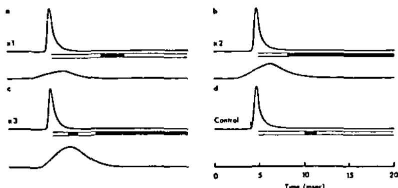


Fig 4 The effect of increasing strength of brief stretch a—c) on recovery of nerve Black bars indicate period of spike response to testing shocks (strength 10 % below resting threshold) c recovery following electrically evoked conditioning spike

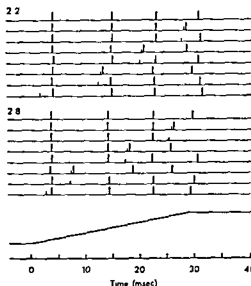


Fig 5 Excitability of afferent nerve during dynamic stretch tested with two strengths (2 2 and 2 8 arbitrary units) of electrical shocks Tests at different delays in successive runs Spikes indicated by thick vertical bars shocks by small thin bars Stretch as indicated below (see text)

dynamic spike was generated during this time It may be concluded therefore that the heightened excitability in this early period was a manifestation of the local current which led up to the generation of a second dynamic spike in the nerve when tretch carried sufficiently far (Ottoson and Shepherd 1968)

The later period appeared to reflect both supernormality of the nerve and the effect of the receptor potential Between these two periods can be seen an interval of relatively low excitability With careful testing this was found to be a regular phenomenon following a brief and relatively strong stretch The explanation for this period of lowered excitability is not obvious It may be noted that the period immediately follows local activity in the nerve and that it is also coincident with the rapid decay of the receptor potential from its dynamic peak and may therefore be related to these events

Prolonged dynamic stretch From the foregoing results it appeared that there are at least three processes which contribute to the heightening of excitability following a single spike elicited by a strong brief stretch 1) supernormality in the wake of the impulse 2) the receptor potential spreading from the nerve terminals and 3) local action currents in the nerve To investigate the relative role these processes played in determining the frequency of spike discharge during dynamic stretch tests of nerve excitability were made in the intervals between the spikes of the response to linearly rising stretch In these experiments it was advantageous to work at lower rates of stretch so that the dynamic response consisted of several spikes with long intervals in between

The result of principal interest from testing in this way was the rise in excitability of the nerve during dynamic stretch This is illustrated in Fig 5 for test shocks of

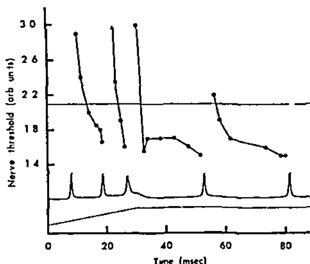


Fig 6 Threshold changes of afferent nerve during response to dynamic and static stretch Ordinate threshold for eliciting an extra spike by a shock to the nerve (arbitrary units) Resting threshold indicated by thin horizontal line Below is shown schematic diagram of spike discharge and time course of stretch

two strengths both well below resting threshold The figure illustrates in schematic form the spike responses elicited by dynamic stretching and the trials in which a test shock just succeeded or just failed to evoke a spike in the aftermath of a preceding dynamic spike If the results are summarized in terms of the apparent refractory period, its duration decreased for successive dynamic spikes, being about 9 msec after the first spike and about 5 msec after the third, using the weaker test shock (2 2) With stronger test shocks (2 8) the apparent refractory period after the first spike was about 4 msec and only slightly less after the second It may be concluded that the stronger shock tested mainly the refractoriness following the previous spike while the weaker shock tested more the increase in excitability leading up to the initiation of the next spike in the discharge These results thus reveal a gradually heightened excitability of the fibre during the dynamic stretch

Experiments were also carried out to compare the excitability of the nerve with different rates of dynamic stretch to the same final amplitude In these experiments it was usually feasible to test only during the interval between the first and second dynamic spikes, at the faster rates there was not time for more spikes to develop The results of these experiments showed that the apparent refractory period shortened with increasing velocity of stretch

The overall time course of the changes in excitability during dynamic stretch could be quantitatively characterized by measuring spike thresholds at different testing intervals Fig 6 shows the data from such an experiment in which thresholds were measured in the spike intervals of the response to a stretch eliciting three dynamic and two static spikes The curve above plots the thresholds as tested by a shock interjected between these spikes After the first spike the threshold drops rapidly the recovery curve falls below the normal threshold into what appears to be

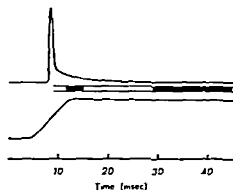


Fig 7 Threshold changes in early period of static stretch Upper trace nerve response with single dynamic spike filled bars, periods of response to testing shock of given strength, lower trace stretch monitor

a period of supernormality This merges into an additional rapid fall in threshold just prior to the onset of the second dynamic spike Following this spike the recovery curve passes more steeply from refractoriness to the generation of the third spike The time course of recovery after the third dynamic spike departs from the dynamic pattern in relation to the changeover from dynamic to static stretch

Static stretch

the experiment illustrated in Fig 6 dynamic stretching was continued beyond the level for generation of the third dynamic spike but not far enough to elicit a fourth spike it then passed into a prolonged static stretch during which the spindle discharged at a lower but steady rate If attention is first directed to the interval between the last dynamic and the first static spike it can be seen that there is a dip in the threshold curve coinciding with the transition from dynamic to static stretch This was followed by a plateau and then a very gradual descent of threshold prior to the generation of the first static spike A similar curve was obtained in the next spike interval except that the dip in threshold was lacking These curves thus reveal two main periods of excitability changes an initial rapid recovery and a subsequent more gradual lowering of threshold

The excitability changes in the period of transition from dynamic to static stretch were also brought out by testing with a shock of given weak strength Fig 7 illustrates the results from an experiment in which dynamic stretch gave rise to one spike only The electrical test shock was effective in two periods (closed bars) The first of these coincided with the termination of dynamic stretching and corresponds therefore with the dip in threshold in Fig 6 while the second covered a period of about 12 msec before the appearance of the first static spike In the time between these two periods the shock failed to elicit a response By comparing Fig 7 with Fig 6 it can be seen that the appearance of the test response is related to the process of increasing excitability preceding the generation of an impulse rather than recovery from refractoriness

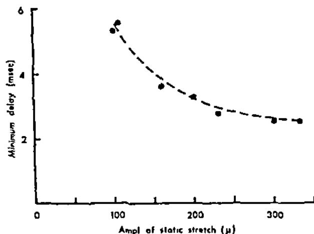


Fig 8 Effect of stretch on minimum delay for eliciting an impulse response in the nerve to a test shock of given amplitude Ordinate delay following initial single dynamic spike Abscissa amplitude of static stretch

The excitability of the fibre increased with increasing level of steady stretch This change was evidenced by measurements of the threshold at a given time during maintained stretch It was also obvious from the gradual shortening of the delay at which a given test shock gave a response One example of this is illustrated in Fig 8 The spindle in this experiment was subjected to a fast rising stretch giving rise to one dynamic spike The excitability in the aftermath of this spike was tested with a shock of a fixed strength As can be seen the minimum latency at which a response could be elicited decreased as stretch was made stronger The change in latency of the response is closely similar to that found under similar conditions using a test stretch (Ottoson *et al* 1968)

When a spike appeared at the very end of dynamic stretch it usually caused a characteristic change in threshold of the nerve One example of this is shown in Fig 9 In this experiment the threshold changes in the transitional interval were first determined when stretch was adjusted so that the second dynamic spike just failed

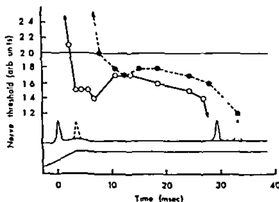


Fig 9 Threshold changes of nerve during stretch with one (open circles) and two (filled circles) dynamic spikes Resting threshold indicated by horizontal line Nerve discharge with (solid line) and without (dashed line) second dynamic spike indicated schematically below together with stretch

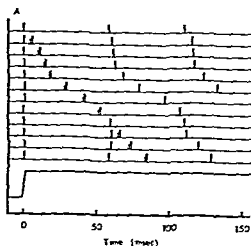
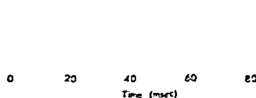


Fig 10 Effect of interjected spike on succeeding impulse discharge Responses to consecutive runs with extra spike interjected at different intervals. *A*, fast rising stretch with one dynamic spike followed by two static spikes

B



B slower rising stretch with two (and on two occasions three) dynamic spikes followed by two static spikes. Note minimal effect on succeeding impulse discharge when interjected spike is at shortest delays but regular effect at longer delays.

to appear By making stretch slightly slower the second spike was made to appear and the threshold curve was determined again. It can be seen that the appearance of the second dynamic spike caused a shift of the threshold curve and a delay of the generation of the static spike There is also a slight shift upward but this may be partially or wholly explained by the slight slowing of the dynamic stretch to allow the second dynamic spike to appear It may be noticed that the period of low threshold, when the second dynamic spike failed to appear persisted into the period of static stretch

While the experiment in Fig 9 suggests that the occurrence of the first static spike is affected by a preceding dynamic impulse this was not the usual finding Often the

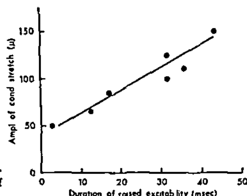


Fig 11 Duration of raised excitability of nerve to given test shock in relation to amplitude of a preceding static stretch

final dynamic spike had little or no effect on the timing of the first static impulse Further results relating to this question were obtained by introducing an extra spike into the discharge evoked by stretch In general the timing of the ensuing impulses was reset by the interjected spike, except when the latter closely followed a dynamic or static spike or when it occurred near the transition from dynamic to static stretch Fig 10 illustrates two such experiments, in A a fast stretch gave rise to a single dynamic spike, in B a slower stretch produced two (and on two occasions, three) dynamic spikes before reaching static level In B (test 7) it may be noted that the occurrence of the first static spike, following three dynamic spikes plus an interjected spike, was only slightly delayed compared to that following only two dynamic spikes These findings are consistent with those obtained when brief stretches were used to elicit the interjected spike (Ottoson *et al* 1968)

With higher levels of static stretch the excitability of the nerve increased but this proved to be difficult to characterize quantitatively At increasing levels of static stretch the impulses were fired at faster rates the corresponding shifts in time of spike occurrence and the modifications of the excitability cycles rendered somewhat ambiguous the results of testing at a given delay Nonetheless many tests at different delays and under different conditions of stretch indicated that there was a general increase in excitability of the nerve with increase in level of static stretch

Aftermath of static stretch During the period immediately following release of static stretch the testing shock revealed a raised excitability of the nerve This is in contrast to the depression in mechanical sensitivity of the spindle as revealed by a test stretch (Ottoson *et al* 1968) This period of raised excitability was independent of any supernormality of a spike near the end of static stretch By using a test shock of subthreshold strength applied at different intervals after release of a moderately strong stretch it was found that the period of raised excitability lasted for about 25 msec for durations of conditioning stretch from 5 to 20 msec With increase in amplitude the duration of the period of raised excitability increased (Fig 11)

Discussion

In the present study the excitability properties of the spindle were studied by using electrical as well as mechanical test stimuli. The conditioning stimuli were also either mechanical or electrical so that four different combinations of paired stimuli arose. In this way the excitability properties of the sensory fibre and its branches could be characterized. The experimental situations arising with these four combinations are schematically summarized in the diagram in Fig. 12. The direct electrical test of the stem fibre is depicted in *a* and *b*. In *a* the conditioning spike travels in antidromic direction while in *b* the nerve is orthodromically invaded. The values obtained are the same in the two situations and define the recovery cycle of the stem fibre. The diagram in *c* and *d* depicts the situation when the properties of the finer intracapsular branches of the fibre were determined. In *c* the impulse evoked by the conditioning electrical stimulus invades the branches antidromically and leaves after it a state of refractoriness, the time course of which is measured by mechanical stimuli giving rise to orthodromic activity. This situation is similar to that arising after conditioning mechanical stimulation *(d)*, in this case the conditioning impulse travels in an orthodromic direction leaving the branches in a state of refractoriness. The values obtained may be assumed to reflect the recovery cycle of the intracapsular branches of the nerve. Apparently these branches have absolute refractory periods about 2 msec longer than the stem fibre. A similar difference in recovery has been demonstrated for the afferent fibre and the endings of rapidly adapting mechanoreceptors in the toad's skin (Lindblom 1958).

As demonstrated in the present study the recovery cycle of the afferent nerve is modified in several ways during and after release of stretch. During dynamic stretch there is a rise in excitability accompanied by a shortening of the recovery cycle for successive spikes. During maintained stretch the threshold of the nerve remains relatively constant and the recovery cycles following successive spikes are more or less identical. It has been shown in previous studies (McReynolds, Ottoson and Shepherd 1966, Ottoson *et al.* 1968) that early in the static phase there is a depression of mechanical sensitivity in terms of the threshold stretch for eliciting a spike. This depression appeared to be related to the fall of the dynamic receptor potential. In this period there often occurs a pause in firing (Shepherd and Ottoson 1965). It would appear that refractoriness of the afferent nerve might be partly responsible for the development of the pause. This possibility was tested in the present study by interjecting an extra spike at different times during the period of transition from dynamic to static stretch. In some cases the first static spike was slightly delayed, in other cases there was no effect. Similar results were found when studying the effect of the first static spike on the succeeding spike. It thus appears that the recovery of the nerve following the last dynamic spike is similar to that after a static spike. This implies that factors other than refractoriness must be relatively more important in relation to the development of the pause. During the period for the appearance of the pause the receptor potential is falling from its peak. It may be suggested that the decay of the receptor potential influences the time course of development of local

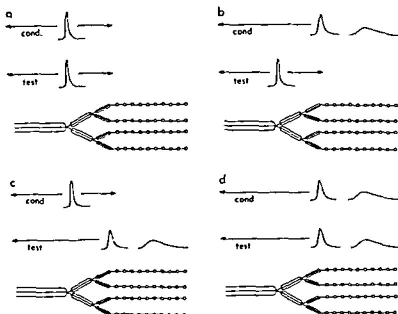


Fig 12 Schematic diagram summarizing four different situations in test of afferent nerve excitability in present study Afferent trunk fibre, branches and sensory endings indicated schematically Electrical shock evokes spikes at first node of stem fibre, a brief stretch produces receptor potential in sensory endings, followed by spike in preterminal myelinated branches Direction of activity indicated by arrows *a—d*, four possible combinations of electrical and mechanical conditioning and testing stimuli (see text)

currents leading up to the generation of the first static impulse and that the pause arises as a result of this

During stretch of the spindle the excitability of the afferent nerve is raised, due to the receptor potential spreading from the nerve endings and graded local potentials developed in the nerve itself Similar changes likely occur in the branches of the nerve except that they have slower recovery cycles and are subjected to greater depolarization from the endings It is interesting to note the striking similarity between the muscle spindle and the touch receptor in the toad s skin with respect to the excitability changes taking place during mechanical stimulation As shown by Lindblom (1963) the excitability of the touch receptor increases in direct relation to the stimulus during mechanical deformation of the skin

To assess the functional coupling between the endings and the stem fibre it is useful to have an estimate for the electrotonic characteristics of the nerve branches Rall (1959 1962 *a*, *b*) has shown that a branching tree can be considered as electrotonically equivalent to a cylindrical extension of a trunk fibre under the assumption of synchronous activity at each level of branching This assumption is valid for the experimental case of antidromic invasion of the branches in the spindle and it must be approximately true for the case of orthodromic activation in view of the sym-

Discussion

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As demonstrated in the present study the recovery cycle of the afferent nerve is modified in several ways during and after release of stretch. During dynamic stretch there is a rise in excitability accompanied by a shortening of the recovery cycle for successive spikes. During maintained stretch the threshold of the nerve remains relatively constant and the recovery cycles following successive spikes are more or less identical. It has been shown in previous studies (McReynolds, Ottoson and Shepherd 1966, Ottoson *et al* 1968) that early in the static phase there is a depression of mechanical sensitivity in terms of the threshold stretch for eliciting a spike. This depression appeared to be related to the fall of the dynamic receptor potential. In this period there often occurs a pause in firing (Shepherd and Ottoson 1965). It would appear that refractoriness of the afferent nerve might be partly responsible for the development of the pause. This possibility was tested in the present study by interjecting an extra spike at different times during the period of transition from dynamic to static stretch. In some cases the first static spike was slightly delayed, in other cases there was no effect. Similar results were found when studying the effect of the first static spike on the succeeding spike. It thus appears that the recovery of the nerve following the last dynamic spike is similar to that after a static spike. This implies that factors other than refractoriness must be relatively more important in relation to the development of the pause. During the period for the appearance of the pause the receptor potential is falling from its peak. It may be suggested that the decay of the receptor potential influences the time course of development of local

view is supported by the fact that under certain conditions one can record small potentials which seem to represent abortive spikes in the branches (*cf* Katz 1950) During stretch this local activity becomes synchronized and the spindle tends to function as a unit (Ottoson and Shepherd 1968)

The excitability changes taking place in the spindle during and after stretch are summarized schematically in the diagram in Fig 13 It can be seen that in general the mechanical sensitivity and the changes in excitability of the nerve are similar except for the early period of static stretch and the period after release of stretch In striking contrast to the profound depression of mechanical sensitivity found in the aftermath of a stretch (Ottoson *et al* 1968) the excitability of the nerve was raised As far as could be determined the raised excitability was related to the receptor potential This would imply that the excitability is a direct function of the depolarization spreading from the sensory endings Although the excitability of the nerve is raised during this time the sensitivity of the spindle to mechanical stretch is lowered The depression in mechanical sensitivity can therefore not be due to a depression in excitability of the nerve This suggests that the depression in the after math of stretch may be due to other factors, which might be of mechanical or metabolic nature

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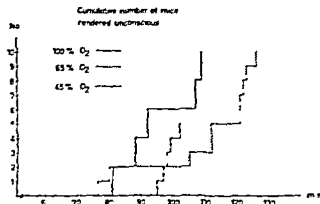


Fig 2 Cumulative number of oxygen fits at an oxygen partial pressure of 4 ata.

clonic as well as a tonic phase They were preceded by a period of low activity prior to a series of almost spastic jumpings around the cage The behaviour of the mice when exposed to 100 % oxygen and 65 % oxygen was identical When exposed to 45 % oxygen the activity of the mice slowed down earlier than with the other mixtures Some of the mice in this experiment also got unconscious without preceding oxygen fits. Thus the activity of these mice gradually decreased until they fell unconscious.

After about 45 min under pressure in the 45 % oxygen mixture experiments the breathing of the mice seemed to be more laborious than in the other experi ts

At decompression which took place within 2 min when 50 % of the mice had become unconscious 25 % in the group with 45 % oxygen mixture), all the unconscious mice were dead or died within a few minutes after arriving to normal atmospheric pressure The surviving mice appeared quite healthy, moving around normally eating and drinking In the 45 % oxygen group 8 were dead when arriving to normal pressure Four more were inflated like balloons from gas in the abdomen and had to be killed All the dead mice also showed varying degrees of hyperinflation which was not seen in the other groups The morbidity of this group (45 % oxygen mixture group due to the decompression was thus greater than in the two other groups although it had been under pressure 7 or 24 min less than the other two groups.

It was also noticed that the first 10 convulsions occurred somewhat earlier with pure oxygen than with the mixtures The difference was probably significant ($P < 0.05$)

Discussion

The results of the present experiments indicate that oxygen toxicity in mice does not increase when oxygen at a partial pressure of 4 ata is mixed with nitrogen at least when using mixtures with 45 % and 65 % oxygen On the contrary the mixing

with 35 % nitrogen somewhat delayed the time for 50 % of the mice getting unconscious at an oxygen partial pressure of 4 ata, although the total pressure in the first case was 4 ata and in the second 6 2 ata At a higher total pressure (8 8 ata), but still 4 ata for oxygen partial pressure, the mice started to become unconscious about 15 min later than in the other series but the number of unconscious mice increased faster once started Because of shortage of the 45 % oxygen gas mixture the exposure had to be terminated when 25 % of the animals had lost consciousness The trend of the curve for the cumulative number of mice rendered unconscious (Fig 1) showed that the time when 50 % of the mice would be unconscious could not be significantly different from that with pure oxygen Consequently it was decided not motivated to repeat this experiment The obvious breathing difficulties the mice showed at the 8 8 ata level (45 % O_2) were most likely due to the increased density of the breathing gas at this pressure The delayed morbidity at the highest pressure might at least partly be due to narcotic influence of the nitrogen in the mixture, apparently delaying the oxygen toxicity The increased density on the other hand might cause hypoventilation with carbon dioxide retention, which later enhanced oxygen poisoning (Bean 1945) This ventilatory insufficiency might appear rather uniformly among the mice, making oxygen poisoning occur more simultaneously among the mice in the 45 % oxygen group than in the pure oxygen and 65 % oxygen group

The first 10 fits occurred a little earlier in the pure oxygen group than in the two other The difference was small and even if statistical analysis revealed that it was probably not due to chance, we do not find it justified to claim that nitrogen counteracts oxygen toxicity

The present experiments thus support the theory of Penrod 1955, that oxygen toxicity depends only on oxygen partial pressure as no increased toxicity appears when the oxygen at constant partial pressure is mixed with nitrogen However it must not be forgotten that the increased density of inert gas — oxygen mixtures at high pressures may produce hypoventilation where carbon dioxide retention increases the risk for oxygen poisoning Results of experiments by Bennet 1967 in the rat support this theory as the toxicity of oxygen increased when it was inhaled with either helium, nitrogen or argon at an total pressure of 18 6 atmospheres Even at this very high total pressure, however, the mean time for oxygen convulsions to occur in the group inhaling oxygen nitrogen mixture, decreased only from 23 8 min to 20 4 min Bennet also showed an increase in cerebral carbon dioxide tension with 12 1 mm Hg in cats, inhaling 2 34 ata O_2 in nitrogen at an total pressure of about 11 ata

The very high pressures Bennet used, however, ought to make his experiments not directly comparable with ours

Histological studies in mice killed by oxygen poisoning during the same conditions as in the present experiments revealed no difference in the effects on adrenergic nerve in the presence of nitrogen (Kaijser and Sachs, to be published)

The results of Lanphier (1955) in man indicating that oxygen is more poisonous

together with nitrogen could probably be explained as an effect of carbon dioxide retention This might have occurred in his volunteers due to an improper breathing equipment, having a dead space of about 500 ml In a later publication Lanphier (1963), in man working with the same equipment, showed that in fact the mean end expiratory P_{CO_2} was 54 mm Hg It was also noticed that it sometimes increased up to 65 mm Hg This CO_2 tension level certainly is capable of giving symptoms from the central nervous system if occurring acutely It also increases the risks for symptoms of oxygen toxicity Furthermore, the subject, who experienced oxygen convulsions after 30 min during work at 100 feet of depth, when breathing a 47 % oxygen in nitrogen mixture, was found to have an abnormal regulation of arterial P_{CO_2} (Lanphier 1963)

Conclusion

The results indicate that it should be worth while to start experiments on humans with oxygen-nitrogen mixtures as diving gas to develop a non decompression diving technique for a depth down to 23 m

It can be calculated that each dive should not exceed 60 min, but that it can be repeated after about 30 min air-breathing at the surface during a whole working day with little risk for bends or oxygen poisoning Naturally new diving tables have to be constructed based on experiments on greater animals and man with proper equipment, as interspecies differences in reaction to oxygen nitrogen mixtures might exist

Both in professional civil diving and military diving such decompression free diving would simplify the procedures and diminish the risks The extra pay for this would be only nominal as oxygen today is a cheap and readily available gas

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The Sympathetic Innervation of the Oviduct in the Rat

By

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Abstract

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The adrenergic innervation of the rat oviduct was studied by fluorescence microscopy The outer half of the circular muscle layer was penetrated by a thin net of adrenergic nerve terminals throughout the isthmus Adrenergic nerve terminals were scarcely seen in the muscular wall of the ampulla In the mesotubarium close to the oviduct a dense net of adrenergic nerve terminals were observed The functional role of the observations is discussed

The mammalian egg is trapped within the oviduct for a period of about 3 days in different species (Anderson 1927) To investigate the morphological prerequisites for a sphincteric function of the isthmus the pattern of the adrenergic innervation of the oviduct was studied in rabbit and man (Brundin and Wirsen 1964 a, b, Sjöberg 1967) In these species the number of adrenergic nerve fibers terminating in the muscular wall of the ampulla was small as compared to the rich supply of adrenergic nerves to the circular muscle of the isthmic part of the oviducts The adrenergic innervation of the cat oviduct shows a similar pattern (Rosengren and Sjoberg 1967) In addition, the extent of the adrenergic innervation in the isthmus of the cow far exceeds that of the ampulla (Brundin and Brundin, to be published) In the rabbit, this rich adrenergic innervation of the isthmus has been correlated to a sphincteric function by experiments *in vivo* (Brundin 1965)

This pattern of distribution of the adrenergic nerves has thus been found in several mammalian species The present investigation was undertaken with the aim to settle if the rat oviduct exhibits the same distribution of adrenergic nerves although the general anatomy of the rat oviduct differs in many respects from that of the species mentioned above (Nilsson and Reinius 1967) Adrenergic innervation to the muscular wall of the rat oviduct has been reported preliminary to occur in the tubo-uterine junction (Norberg and Fredricsson 1966)



Fig 1 Oviduct isthmus rat In the outer part of the circular muscle layer (A) fluorescent adrenergic nerve terminals are seen Such terminals are also found in the mesotubarium (B) and surrounding small blood vessels (C) Epithelial autofluorescence is also seen Fluorescence micrograph, ×80

Material and methods

Female white rats of the Wistar strain were used in which full vaginal opening had occurred (weight 250—300 g) The animals were rapidly decapitated in ether anesthesia and their genital organs were removed for final dissection under microscope Within 5 min after the [illegible] ducts were instantly frozen in propene/ [illegible] mens were dried *in vacuo* and treated [illegible] k *et al* (1962) Paraffin sections (8μ) [illegible] routine filter equipment (Falck and [illegible]

Results

In sections from the isthmic part of the rat oviduct (Fig 1) a delicate net of adrenergic nerve terminals was seen to intermingle with the muscle cells of the outer half of the circular layer while the inner portion of the circular muscle was free from adrenergic innervation In the epithelium a brownish auto-fluorescence was constantly observed No extravascular adrenergic innervation to the mucosa was seen. In the mesotubarium adjacent to the oviduct, a great number of adrenergic nerve terminals was seen Many of these terminals innervate the vessels supplying the isthmus but some of the adrenergic nerve filaments seen in the mesotubarium *could not* be connected to any vessels This finding might imply an adrenergic innervation of smooth muscles in the mesotubarium, close to the oviduct (*cf* Fig 1)



Fig 2 Oviduct ampulla (A) and isthmus (B), rat Fluorescent adrenergic nerve terminals are seen in the outer muscle layer of the isthmus (C) but in the ampulla they are mainly restricted to the blood vessels (D) In the mesotubarium adrenergic terminals innervate both vessels (E) and extravascular smooth muscle (F) Fluorescence micrograph ×150

In the ampulla (Fig 2) any extravascular innervation to the thin muscular wall is scarce if at all present The scarcity of adrenergic nerve terminals to the musculature of the ampullary wall was a constant observation

Discussion

The present results indicate that the total number of adrenergic nerve fibers innervating the isthmic circular muscles far exceeds that of the muscular wall of the ampulla Whether this difference reflects the greater mass of the isthmic muscle or, in addition means a denser innervation of the isthmic than the ampullary muscle cells can not be stated on basis of the present results Anyway the greater number of adrenergic nerve fibers in the isthmus must be functionally important The postovulatory constriction of the isthmus is necessary to retain the fertilized eggs of the rat in the ampulla up to the blastocyst stage (Alden 1942) The strength of this constriction in rabbits has been shown to be directly proportional to the impulse frequency during stimulation of the adrenergic nerves to the oviduct (Brundin 1965) The restriction to the isthmic part of the adrenergic smooth muscle innervation of the rat oviduct indicates that the postovulatory constriction of the isthmus could be caused by adrenergic stimulation of the isthmic circular muscles also in this species

The abundant occurrence of adrenergic nerve terminals in the mesotubarium has not been described earlier in mammals The smooth muscle fibers in the mesotubarium of the rabbit are very sensitive *in vitro* to noradrenaline which causes abrupt constriction (Brundin, unpublished). The coiled shape of the oviduct of the rat (*cf* Fig 2) increases the possibilities for contractions in the smooth muscles of the mesotubarium to minimize the luminal passage through the isthmus by causing kinks on the oviduct. Such an additional mechanism for occlusion of the rat oviduct might explain the observation that the number of adrenergic nerve terminals in the rat isthmus are fewer than those found in *e g.* rabbit and man, where the oviduct has a more straight course

This investigation was supported by the Swedish Medical Research Council (Project nr B68-14X-2323-01).

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Effects of Reserpine on the Noradrenaline Content of Atrophied Rat Salivary Glands

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Abstract

JONASON, J, *Effects of reserpine on the noradrenaline content of atrophied rat salivary glands* Acta physiol scand 1969 *75* 73—77

The noradrenaline content was determined in intact and atrophied rat salivary glands at different time intervals after reserpine treatment both with and without previous monoamine oxidase [illegible]

produced a much slower noradrenaline reduction rate. After this treatment a slower decrease was observed in the atrophied glands than in the intact ones The data in this study indicate that most of the noradrenaline after reserpine treatment is metabolized by means of intraneuronally localized monoamine oxidase

After ligation of the excretory ducts of rat salivary glands, the gland cells atrophy with a concomitant decrease in weight (Junqueira 1951, Bhaskar, Bolden and Weinmann 1956, Standish and Shafer 1957) The monoamine oxidase (MAO) activity is reduced to about the same extent as the weight loss or by more than 60 per cent as found both biochemically and histochemically (Almgren *et al* 1966) On the other hand, the adrenergic nerve terminals of the salivary glands do not show a corresponding atrophy The noradrenaline (NA) content per gland is almost unchanged biochemically and the network of the NA terminals is much denser than in normal glands (Anden, Norberg and Olson 1966) It is well known that after reserpine treatment there is an increased catabolism of catecholamines by MAO (Carlsson 1965) Whether this increased deamination takes place intra neuronally or extraneuronally is not known In the present investigation this matter has been studied by measuring the rate of disappearance of NA after reserpine treatment in atrophied salivary glands (that is glands with no extraneuronal MAO) and normal ones, both with and without previous MAO inhibition

Material and Methods

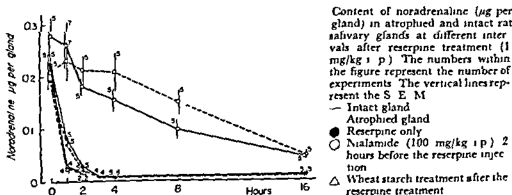
Adult hooded and Sprague Dawley rats of both sexes weighing about 200 g were used Under pentobarbital sodium anesthesia (40 mg/kg i p) the excretory ducts of the left submaxillary and sublingual glands were ligated near the hilum Fourteen days after the operation, when atrophy of the left gland was completed, reserpine was injected intraperitoneally in a dose of 1 mg/kg body weight About half of the animals were pretreated with nialamide (100 mg/kg i p) two hours before the reserpine injection The rats were sacrificed by a blow on the head at different intervals after the reserpine administration The submaxillary plus the sublingual glands on each side were removed as soon as possible, weighed and homogenized in 10 ml 0 4 N perchloric acid by an "Ultra-Turrax' homogenizer The NA was determined spectrophotofluorometrically after cation exchange chromatography (Bertler, Carlsson and Rosengren 1958) If the glands on the ligated side weighed more than 50 per cent of those on the intact side the values were discarded

A few rats were given dry wheat starch continuously in the mouth for 1 or 2 hours after the reserpine injection This procedure has been shown to increase the impulse flow of the adrenergic nerves to the salivary glands (Hillarp 1949)

The values are expressed as means ± the standard errors of the mean (S E M) Generally, the P values are calculated by Student's t test

Results

The results are presented in Fig 1 In the rats not pretreated with nialamide there was no significant difference in the content of NA between the intact and the atrophied glands at the time of reserpine administration After reserpine alone, the NA of both types of glands disappeared at the same rate The minimum level was achieved after about 4 hours and this low level was maintained for the rest of the investigated time intervals The loss of NA was accelerated in the rats given dry wheat starch without any difference between the intact and the atrophied glands The decrease of NA after reserpine plus dry wheat starch was significantly larger than that after reserpine alone one hour after reserpine administration ($P < 0\ 025$)



Content of noradrenaline (µg per gland) in atrophied and intact rat salivary glands at different intervals after reserpine treatment (1 mg/kg i p) The numbers within the figure represent the number of experiments The vertical lines represent the S E M
— Intact gland
Atrophied gland
● Reserpine only
○ Nialamide (100 mg/kg i p) 2 hours before the reserpine injection
△ Wheat starch treatment after the reserpine treatment

Obviously the impulse flow in the adrenergic nerves influences the NA reduction, a phenomenon observed earlier by Sedvall (1964)

Pretreatment of the rats with the MAO inhibitor nialamide did not significantly increase the content of NA in the two types of glands However, after this pretreatment reserpine produced a much slower disappearance of the salivary gland NA in both the atrophied and the intact glands This difference in NA content between reserpine treated animals and animals treated with nialamide plus reserpine, is significant at $P < 0\ 01$ after 1 hr, $P < 0\ 001$ after 2 and 4 hrs and $P < 0\ 005$ after 16 hrs Furthermore, there appears to be a difference in the NA content between atrophied and intact glands after nialamide plus reserpine Initially, the NA level of the intact glands is higher than that of the atrophied ones ($P < 0\ 005$) but from the beginning the disappearance rate of NA is somewhat higher in the intact glands This phenomenon results in significantly lower NA levels in the intact glands compared with the atrophied glands after 4 and 8 hrs at $P < 0\ 05$ (The results were analyzed by the difference method of Davies, 1949) After 16 hrs there is no difference in the NA level of the two types of glands

In another series of experiments normal animals were pretreated with nialamide in a small dose (20 mg/kg i p) two hours before reserpine administration The NA content was determined in one of the submaxillary salivary glands and the MAO activity in the other gland (using the radioactive tyramine method described by Almgren *et al* 1966) In those animals which showed an inhibition of the MAO activity in the salivary gland by 50 % to 80 %, the NA content in the contralateral gland was (mean ± S E M) 0 196 μg ± 0 069 after 1 hr (3 values), 0 213 μg ± 0 028 after 2 hrs (3 values), 0 089 μg ± 0 022 after 3 hrs and 0 049 μg ± 0 007 4 hrs after reserpine administration (compare these data with Fig 1) The NA reduction rate after reserpine is obviously decreased by inhibition of MAO in the whole gland by 50 %—80 %

Discussion

The present results show that there is no significant difference between intact and atrophied salivary glands in the rate of the NA disappearance after reserpine treatment Thus finding suggests that there is no severe disturbance of the blood circulation in the atrophied glands In both cases MAO inhibition significantly reduces the rate of decrease This prevention of the reserpine effect on the NA by a MAO inhibitor suggests that after reserpine treatment most of the NA is metabolized by means of MAO (Carlsson *et al* 1957, Malmfors 1965) It is to be noted that the MAO activity is reduced by at least 60 per cent in atrophied glands, and this loss of MAO activity occurs extraneuronally (Almgren *et al* 1966) It has been pointed out by other investigators that the oxidative deamination of NA by means of MAO mainly occurs intraneuronally (Carlsson *et al* 1957, Spector *et al* 1960 Carlsson 1960, Carlsson and Hillarp 1962, Kopin 1964) but the evidences in favour of this is of a more or less indirect nature We can see that if almost all

of the extraneuronal MAO is selectively destroyed the disappearance rate of NA after reserpine is not decreased These data strongly support the view that the oxidative deamination of NA after reserpine treatment occurs intraneuronally

Furthermore it is possible that the remaining enzyme activity is sufficient from a functional point of view It is known that inhibition of MAO to at least 85 % is necessary to produce an increased amine level in the brain (*cf* Pletscher, Gey and Zeller 1960, p 442) However in the atrophied glands there is a selective loss of almost all extraneuronal MAO, the intraneuronally localized part of the enzyme being intact After nialamide inhibition of MAO activity in the whole gland by 50 %—80 % the NA reduction rate after reserpine is decreased (see Results) In contrast the NA disappearance rate after reserpine in atrophied glands is not affected, even though the MAO activity is reduced to about the same extent Therefore, the data indicate that the oxidative deamination of NA after reserpine treatment occurs intraneuronally

A basic difference between extraneuronally and intraneuronally localized MAO is that at the level of the nerve cell membrane, there is an amine concentrating mechanism "the membrane pump which concentrates the substrate for MAO within the nerve (Hamberger *et al* 1964, Hillarp and Malmfors 1964, Muscholl 1965, Malmfors 1965, Carlsson and Waldeck 1965 a, b, c) This mechanism increases the relative importance of this intraneuronally localized enzyme The present data support the view that the MAO within the nerve is of greatest importance for the NA reduction after reserpine

It seems probable that after MAO inhibition followed by reserpine treatment the NA leaves the adrenergic nerves and is 3-0 methylated by means of the enzyme catechol-0-methyl transferase or is transported away unchanged by the blood An interesting phenomenon is that the NA reduction is significantly slower in the atrophied glands compared to the intact ones after nialamide plus reserpine The reason for this might be loss of the enzyme catechol-0 methyl transferase as a consequence of parenchymal atrophy If this enzyme is localized within or at the surface of the parenchyma cells a decreased NA reduction after nialamide plus reserpine would be expected in the atrophied glands In fact we have been able to demonstrate a severe reduction of the activity of catechol-0-methyl transferase in the salivary glands after atrophy by incubating salivary gland slices with labeled dopamine or noradrenaline Jonason to be published)

The present data indicate that the reserpine-sensitive storage mechanism of the adrenergic nerves remains intact after parenchymal atrophy This seems to be true also of transmitter release by the nerve impulse It is true that the adrenergic nerves of the atrophied salivary glands have a reduced ability to take up circulating monoamines, but this phenomenon may be explained by a loss of extraneuronal binding sites from which the nerves appear to take up amines (Almgren Andén and Waldeck 1965)

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fat accumulation are increased uptake of plasma free fatty acids by the liver or decreased oxidation of fatty acids in the liver The present work was done to study these possibilities

Materials and methods

^{3}H-oleic acid (The Radiochemical Centre, Amersham, England) was chosen as a suitable

1965)

acid columns into two fractions one containing mainly triglyceride and the other mainly phospholipid (Olivecrona 1962) Glyceride glycerol was determined by the method of Van Handel and Zilversmit (1957) The remains of the rats were digested in 20 % ethanolic KOH and the fatty acids extracted as described previously (Goransson and Olivecrona 1964) Aliquots of the liver lipid extract, the glyceride and phospholipid fractions and the fatty acids extracted from the carcass digest were assayed for radioactivity in a Packard liquid scintillation spectrometer Quenching was corrected for by use of an internal standard

Results

The results are shown in Table I The total liver radioactivity was highest in the ethanol treated groups intermediary in the glucose treated groups and lowest in the fasted groups The difference between the ethanol treated groups and the control groups was statistically significant ($p<0.01$) The differences between the groups treated with and the groups not treated with nicotinic acid in each nutritional state were not statistically significant The carcass radioactivity was similar in the ethanol treated and the fasted groups, but tended to be higher in the glucose treated groups However the difference was not statistically significant The plasma free fatty acid concentration was similar in the ethanol treated and the fasted groups not given nicotinic acid and significantly lower ($p<0.01$) in the corresponding glucose treated group In all nutritional states nicotinic acid lowered the plasma free fatty acid concentration The liver glyceride concentration was considerably elevated in the ethanol treated group In all cases the liver glyceride concentration was decreased by nicotinic acid treatment

Discussion

The data show that the fraction of the plasma free fatty acid flux that was incorporated into the liver glycerides was increased by ethanol This could be explained by an increase in the uptake of free fatty acids from plasma as suggested by Nikkilä

TABLE I Radioactivity in liver and carcass lipids 5 min after I V injection of albumin bound ³H oleic acid, and concentration of liver and plasma lipids

Treatment	Radioactivity (% of injected dose)			Lipid concentration	
	Liver glycerides	liver phospholipids	carcass	liver glycerides µmole/liver and 200 g body weight	plasma free fatty acids µmole/ml
Ethanol	35 2 ± 3 8	2 3 ± 0 5	37 6 ± 5 2	156 1 ± 31 2	1 02 ± 0 08
Ethanol + nicotinic acid	27 9 ± 2 9	6 2 ± 0 7	31 3 ± 2 2	37 0 ± 2 4	0 57 ± 0 05
None	18 5 ± 4 0	3 5 ± 0 4	40 9 ± 6 5	71 9 ± 9 1	1 05 ± 0 18
None + nicotinic acid	12 7 ± 1 9	5 8 ± 1 4	33 3 ± 7 2	18 6 ± 6 6	0 61 ± 0 14
Glucose	19 9 ± 1 7	4 4 ± 0 8	44 2 ± 9 1	53 3 ± 4 6	0 76 ± 0 06
Glucose + nicotinic acid	15 9 ± 1 7	6 3 ± 1 1	47 6 ± 7 5	37 2 ± 12 3	0 54 ± 0 05

Female rats (140—170 g) were given ethanol (6 g/kg B W), isocaloric amounts of glucose or nothing 6 hrs before they were killed Nicotinic acid was given as 6 hourly subcutaneous injections of 15 mg each All values are mean ± S D of 6 animals

and Ojala (Nikkila and Ojala 1963) However, much evidence shows that the uptake of plasma free fatty acids by the liver is not changed by nutritional or hormonal factors but is a direct function of the plasma free fatty acid concentration and blood flow (Fine and Williams 1960, Morris 1963) We therefore feel that it is more probable that the fraction of the plasma free fatty acid flux taken up by the liver was similar in all groups and that the different amounts of radioactivity found in the liver were due to differences in oxidation of plasma free fatty acids after uptake into the liver According to this view only little oxidation occurred in the livers of the ethanol treated rats whereas a considerable fraction of the free fatty acids taken up was oxidized in the livers of the two other groups This view is further strengthened by the fact that the liver radioactivity was similar in the ethanol treated rats in the present experiment and in the carbohydrate-refed rats in a previous study (Goransson and Olivecrona 1965) Since ethanol is oxidized mainly by the liver the feeding of ethanol would not be expected to influence fatty acid oxidation in the extrahepatic tissues The present data show similar radioactivity in the carcass of the ethanol treated and the fasted rats This is in agreement with our view that the tissue distribution of the plasma free fatty acid flux was not changed by ethanol

Treatment with nicotinic acid reduced the plasma free fatty acid concentration and prevented the increase in liver triglyceride induced by ethanol This is in agreement with the studies of Brodie and Maickel (Brodie and Maickel 1963) who showed that interference with free fatty acid mobilization reduces the ethanol induced

fatty liver Nicotinic acid treatment did not significantly change the total incorporation of label into liver lipids, but caused a shift of radioactivity from the glyceride to the phospholipid fraction Probably this was a result of an increased specific radioactivity of the plasma free fatty acids rather than a real increase in the fatty acid incorporation into the phospholipid fraction

Our data show that hepatic oxidation of plasma free fatty acids is probably decreased by ethanol and that the acute ethanol induced fatty liver can be prevented by nicotinic acid probably due to a decrease in the mobilisation of free fatty acids from adipose tissue We suggest that the acute ethanol induced fatty liver in the rat is caused by a decreased hepatic oxidation of plasma free fatty acids taken up at a normal rate

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Blood Flow, Oxygen Uptake and Lactate Production in the Forearm during Exercise Induced by Median Nerve Stimulation

By

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Received 16 April 1968

Abstract

LANDIN, S. and J. WAHREN. *Blood flow, oxygen uptake and lactate production in the forearm during exercise induced by median nerve stimulation.* Acta physiol. scand. 1969. 75. 82—91.

A technique is described for percutaneous stimulation of the median nerve and the induction of a standardized involuntary forearm exercise. The electrically induced exercise is compared with voluntary forearm exercise in 14 healthy subjects at work intensities of 4, 6 and 8 kpm/min. Blood flow, oxygen uptake and lactate production were found to be higher for electrically induced exercise than for voluntary, especially when the duration of the stimulus was increased from 0.1 sec to 0.3 sec. The increases in these parameters in relation to work intensity were steeper for electrically induced exercise than for voluntary. The differences are partly attributed to the exercise induced by electrical stimulation having a larger component of energy expenditure in static work from the activation of muscles that do not participate in finger flexion.

The blood flow and metabolism of isolated or in situ muscles activated by nerve stimulation, has been extensively studied in animals (Kramer, Obal and Quensel 1939, Stainsby and Otis 1964). Such studies have been performed on normal muscles as well as on muscles rendered atrophic by denervation or tenotomy. Denervated muscle is reported to exhibit an increased blood flow and oxygen uptake as well as an increased utilization of glucose as compared to healthy muscles (Bass and Hudlicka 1961).

Similar studies in man have not been published to date. The technique outlined by Wahren (1966) however permits quantitive measurements of blood flow in the human forearm during exercise by continuous infusion of dye into the brachial artery. Blood samples can be obtained simultaneously for the analysis of muscle arterio-venous differences of various metabolites. This communication describes a technique, based on electrical stimulation of the median nerve, for inducing involuntary and standardized exercise of the human forearm muscles. The procedure

which is independent of the patient's capacity for voluntary muscle contraction and active co-operation, has been used to study the effect of voluntary and involuntary forearm exercise on blood flow, oxygen uptake and lactate production of the forearm muscles

Material and methods

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The subjects came fasting to the laboratory in the morning Teflon catheters were inserted percutaneously into the brachial and radial arteries and into a deep and a superficial forearm vein

Rhythmic forearm exercise was performed in the supine position with the arm horizontal and at an angle of ca 45° to the body The elbow was extended and the volar side of the [illegible]

technique, based on continuous infusion for one minute of dye solution (indocyanine green in 5 per cent dextran solution) into the brachial artery

Electrical median nerve stimulation Two connected Grass stimulators (type S 4) and a stimulus isolation unit (Grass, type SIU-4) were used for the stimulation The stimulators were calibrated repeatedly between the experiments The optimal point of stimulation of the median nerve was localized in each subject, usually on the ulnar side of the cubital fossa or on the distal, ulnar side of the upper arm The cathode was applied there and the anode on the volar side of the ipsilateral wrist. A low resistance electrode paste and metal electrodes were applied after scrubbing the skin

The stimuli were given at a frequency of 90 min, each stimulus having a duration of 0 1 or 0 3 sec and consisting of square waves of 1 msec duration with a frequency of 30 per sec Experiments were performed at work intensities of 4 6 and 8 kpm/min It is known that 4 kpm/min is the lowest work intensity at which deep venous blood is not contaminated with blood from superficial forearm veins (Idbohrn and Wahren 1964) An increase in work intensity (raised spring tension of the hand ergometer) during electrically induced exercise was accomplished by raising the strength of the stimulation maintaining its frequency unchanged and at the same time increasing the spring tension of the hand ergometer The strength of the stimulus varied between the subjects but was always about 1 1/2—2 times the rheob[illegible]

Electromyographic recordings were made with a Grass P 5 preamplifier and a Tektronix oscilloscope type 502 Concentric needle electrodes and surface electrodes were used

Blood analyses

Blood sampling for analysis of arterio-venous (deep vein = dv) oxygen difference and la[illegible] concentrations was made at 4 and 8 min during each exercise period

Oxygen saturation was determined spectrophotometrically by a slightly modifi[illegible] method described by Drabkin (1950)

Hemoglobin concentration was analyzed by the cyanmethemoglobin technique (Dr[illegible] and Austin 1935) using a reagent and standard from Hycel Inc

Hematocrit was measured and corrected for trapped plasma (1 3 per cent [illegible] Garby and Vuille (1961), using a high speed microcapillary hematocrit [illegible] 12,500 rpm)

Lactate concentration was analyzed with an enzymatic method based [illegible] described by Hohorst 1962) and Lundholm Mohme-Lundholm and S[illegible]

Indocyanine green concentration was analyzed spectrophotometrically [illegible] spectrophotometer A calibration curve was made for each subject [illegible] 8 mg/l

For error of methods see Wahren (1966

Results

Technique of stimulation

All the subjects considered that the stimulation was perfectly tolerable provided that the point chosen over the median nerve really was the optimal one

Trials were made with different types of stimulation The frequency finally chosen was 90 min If the frequency was too low e g 60 min it was necessary to increase the strength or prolong the duration of each contraction in order to obtain a constant specific minimum work intensity Pain or some other reason often made it impossible to perform the increased work per contraction While it was possible to maintain a stimulation frequency of 120 min this also proved unsuitable for several reasons The hand ergometer did not always return to the resting position between contractions and the subjects reported subjective discomfort on a couple of occasions In addition it was difficult to perform the corresponding voluntary work sufficiently accurately at such a high rate of contraction

Trials were also made with different durations of the stimulus It was found that the subjective discomfort increased with increasing duration Some investigations were conducted with the longer duration 0 3 sec whereupon most of the subjects reported moderate pain at the point of stimulation and in the forearm particularly towards the end of the work A further disadvantage of a duration of 0 3 sec was that the hand ergometer did not always return to the initial position between the contractions The results from such experiments are not included below

As mentioned above each stimulus had a duration of 0 1 or 0 3 sec being made up of square waves of 1 msec duration with a frequency of 30 sec If the frequency was increased to 40—60 per sec there was generally a moderate to large initial increase in the muscle strength developed After one or at most 2 min however the musculature became fatigued so that all work usually stopped entirely for about 1/2 min

The strength of the stimulus varied between the subjects as mentioned above The rheobase averaged 30 V (range 25—40) maximal work being obtained when the voltage was about twice the rheobase or a little higher This type of stimulation was not painful

Electromyographic recordings The muscle response to the various strengths of stimulation was controlled electromyographically (Fig 1) The subjects were instructed to relax in order to avoid the superimposition of voluntary activity upon the electrical stimulus No appreciable indirect responses of the H or F type (Magladery and McDougal 1957) were observed at the strength employed An F response appeared regularly however at somewhat higher stimulation strengths though its amplitude was small in relation to the direct muscle response It therefore seems that there was no or very little contamination of the muscular response from secondary stimulation effects

Electromyographic recordings from the extensor muscles in the dorsal part of the forearm were undertaken during voluntary work with and without the arm being

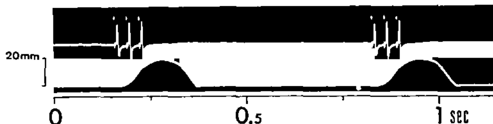


Fig 1 Upper tracing shows EMG from forearm flexor muscle (cutaneous electrode) during electrically induced exercise at 8 kpm/min Stimulation frequency 90 min, duration of each stimulus 0 1 sec Lower tracing is registration of the movement of the hand ergometer handle

placed in the plastic sheath described above It will be seen from Fig 2 that less activity is initiated in the extensor muscles when voluntary work is performed with the arm in the sheath (as in the subsequent experimental conditions) that with the arm free When voluntary work was performed with the arm in the sheath, the electromyographhic activity in the extensor musculature did not deviate substantially from that recorded during electrical stimulation of the median nerve with the arm in the sheath

Different types of exercise

A *Varying the stimulus duration* (Fig 3, Table I and II) In 8 subjects experiments were made with stimuli of 0 1 and 0 3 sec duration in electrically induced exercise These types of exercise were compared with voluntary exercise, all the experiments being performed at a work intensity of 4 kpm/min. Each subject exercised for three separate periods of 8 min with a rest interval of 45—60 min

Blood flow was larger during electrically induced exercise than during voluntary exercise while a stimulus of 0 3 sec duration resulted in a higher blood flow than one of 0 1 sec The blood flow tended to rise between 4 and 8 min during electrically induced exercise ($p<0\,05$) but not during voluntary exercise

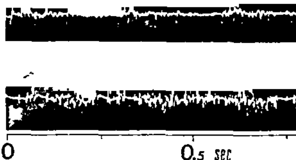


Fig 2 EMG from forearm extensor muscle during voluntary exercise at 8 kpm/min (needle electrode) Upper tracing recorded during exercise with the fore arm placed in a plastic casing and the lower with the forearm free

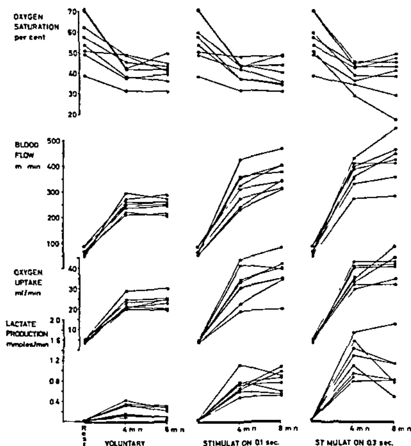


Fig 3 Deep venous oxygen saturation blood flow oxygen uptake and lactate production of the forearm during voluntary exercise at 4 kpm/min and during electrically induced exercise with stimuli of 0 1 sec and 0 3 sec at the same work intensity The order of the exercise periods was randomized and there was a rest interval of 45—60 min between them Values at rest were measured before the first exercise period only

Oxygen uptake The arterio-venous oxygen differences did not differ significantly between *electrically induced and voluntary exercise Calculated oxygen uptake* was higher during both durations of electrical stimulation than during voluntary exercise, the value for 0 3 sec being slightly but not significantly higher in its turn than that for 0 1 sec The time courses for oxygen uptake during the different types of exercise were similar to those for blood flow

Lactate production was markedly lower in voluntary exercise than during electrical stimulation, the lower calculated values being associated with lower venous-arterial lactate concentration differences as well as lower blood flows Moreover, an 0 3 sec stimulus gave rise to a larger lactate production at 4 min than an 0 1 sec stimulus ($p<0\,01$) At 8 min exercise this difference was no longer significant since the lactate production with an 0 3 sec stimulus had then decreased markedly

TABLE I M ± SD (n = 8) for blood flow (F, ml/min) arterio-venous oxygen difference (a—dv) O_2 ml/l oxygen uptake (Q_{O_2} ml/min) venous arterial lactate difference $(dv—a)_{lactate}$ mmoles/l and lactate production ($Q_{lactate}$ mmoles/min) at rest and during exercise (4 kpm/min) induced by electrical stimulation (0 1 and 0 3 sec) and during voluntary exercise

	Rest	Stimulation 0 1 sec		0 3 sec		Voluntary	
		4 min	8 min	4 min	8 min	4 min	8 min
F	66±15	317±65	369±55	370±51	422±90	248±28	249±28
(a—dv) O_2	80 1	106 7	105 8	104 1	104 2	100 2	103 9
	±14 5	±12 3	±16 7	±10 6	±13 0	±12 4	±13 1
Q_{O_2}	4 1	32 1	37 1	36 3	41 4	22 8	23 3
	±0 7	±8 6	±8 6	±4 7	±6 3	±2 6	±3 7
$(dv—a)_{lactate}$	0 13	2 52	2 31	3 78	2 88	1 04	0 74
	±0 13	±0 53	±0 67	±1 51	±2 11	±0 57	±0 47
$Q_{lactate}$	0 01	0 71	0 76	1 21	1 01	0 23	0 16
	±0 01	±0 19	±0 21	±0 36	±0 46	±0 13	±0 11

TABLE II M ± SD (n = 8) for intraindividual differences in blood flow (F, ml/min) arterio-venous oxygen difference (a—dv) O_2 ml/l oxygen uptake (Q_{O_2} ml/min), venous arterial lactate difference $(dv—a)_{lactate}$ mmoles/l and lactate production ($Q_{lactate}$ mmoles/min) between different types of exercise Measurements were made at 4 and 8 min The probability that the differences were caused by random factors are indicated $p<0.05$ $p<0.01$ and $p<0.001$

	Time during exercise	0 1 stim vol	p	0 3 stim 0 1 stim	p	0 3 stim vol	p
F	4 min	69±79	<0 05	53±45	<0 01	122±69	<0 01
	8 min	120±61	<0 001	49±40	<0 05	173±98	<0 01
(a—dv) O_2	4 min	6 6±7 9	<0 05	−2 6±17 9		4 0±19 5	
	8 min	1 9±13 3		−0 7±30 0		1 2±23 0	
Q_{O_2}	4 min	9 3±7 9	<0 05	4 2±8 5		13 5±6 3	<0 001
	8 min	13 7±7 0	<0 001	4 1±5 6		18 2±6 2	<0 001
$(dv—a)_{lactate}$	4 min	1 48±0 84	<0 01	1 22±1 55		2 70±1 55	<0 01
	8 min	1 57±0 63	<0 001	0 50±2 41		2 18±2 10	<0 05
	4 min	0 48±0 29	<0 001	0 50±0 38	<0 01	0 98±0 38	<0 001
$Q_{lactate}$	8 min	0 60±0 23	<0 001	0 21±0 62		0 71±0 31	<0 01

B *Increasing the work intensity* (Fig 4 Table III) In 6 subjects, experiments were made with electrically induced 0 1 sec stimulation and voluntary exercise at two different work intensities each All the subjects performed exercise at 4 kpm/min for 8 min after which four subjects exercised for a further 8 min at 8 kpm/min

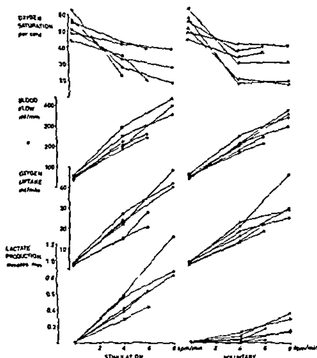


Fig 4 Deep venous oxygen saturation blood flow oxygen uptake and lactate production of the forearm during exercise at 4, 6 and 8 kpm/min induced by median nerve stimulation Measurements were made at 8 min exercise and corresponding values for voluntary exercise are given *Individual symbols are used for* the results from different subjects.

TABLE III Mean values and range (n = 6) for blood flow (F, *ml/min*), arterio venous oxygen a)$_{lactate}$, *mmoles/l*) and lactate production ($Q_{lactate}$, *mmoles/min) at rest and at*

	Rest	El stimulation 0 1 sec			
		4 kpm/min		8 kpm/min	
		4 min	8 min	4 min	8 min
F	53 45–63	228 190–274	230 190–294	380 375–390	396 358–434
(a—dv) O_2	77 6 58 6–92 8	97 7 77 4–124 7	99 8 82 7–116 5	114 6 97 2–12 82	111 2 101 4–121 0
$\dot{Q}_{O_2}$	2 9 2 2–3 5	20 6 15 2–24 8	21 3 15 0–27 4	41 4 34 3–48 4	41 0 40 2–41 8
(dv—a)$_{lactate}$	*0 10* 0 04–0 15	*2 79* 1 84–3 80	*2 41* 1 61–3 32	*3 27* 2 22–4 65	*2 06* 2 04–2 07
$Q_{lactate}$	0 01	0 56 0 31–0 68	0 47 0 29–0 59	0 99 0 77–1 21	0 85 0 82–0 87

the other two subjects at 6 kpm/min The two periods at different work intensities were performed without any interval for rest A rest pause of 45—60 min separated the voluntary and electrically induced exercise

Blood flow during exercise showed an approximately linear relationship to work intensity The rise in blood flow was slightly steeper during electrically induced exercise than during voluntary, all individual flow values being slightly higher during stimulation (*cf* Fig 4)

Oxygen uptake The arterio-venous oxygen difference did not differ significantly between electrically induced and voluntary exercise Calculated oxygen uptake was found to be linearly related to work intensity for both types of exercise, with a slightly steeper rise in oxygen uptake during stimulation than during voluntary exercise

Lactate production rose markedly more rapidly during stimulation and increasing work intensity than during voluntary exercise The higher calculated values were associated with higher lactate venous arterial differences during stimulation and only to some extent with increased blood flow The rise in lactate production during electrically induced exercise of rising intensity was approximately linear, while an approximately exponential relationship was observed during voluntary exercise

Discussion

The present study was carried out in order to find a procedure for inducing involuntary and standardized muscular exercise for the future study of muscle circulation and metabolism in patients with motor disturbances The forearm was

difference (a—dv) O_2 oxygen uptake (Q_{O_2} ml/min) venous arterial lactate d fference (dv—
4 and 8 kpm/min exercise electrically induced and voluntary

Voluntary exercise			
4 kpm/min		8 kpm/min	
4 min	8 min	4 min	8 min
214 175 286	204 170—250	315 280—358	342 291 377
92 8 73 0—109 0	93 1 85 1—105 8	108 9 85 0—149 6	99 7 89 21—31 6
18 0 13 2—23 7	17 5 13 4—22 9	31 9 27 1—40 1	32 5 24 8—46 6
0 54 0 114— 06	0 42 0 02 1 01	0 97 0 47—1 47	0 72 0 42—1 12
0 10 0 02—0 17	0 07 0—0 16	0 28 0 51—0 45	0 23 0 13—0 37

found to be well suited for such studies, the median nerve being accessible for electrical stimulation percutaneously and it being possible to catheterize both the supplying artery and the deep forearm veins. At rest, blood draining the forearm muscles almost exclusively can be obtained from the deep veins when the hand circulation is occluded by a cuff at the wrist (Mottram 1955, Coles *et al.* 1958). During rhythmic forearm exercise at 4 kpm/min or more the deep venous blood has been found to derive practically entirely from forearm muscle even when the hand circulation is free (Idbohrn and Wahren 1964).

Electrical stimulation of the median nerve was well tolerated by the subjects and was easy to carry out when the exercise was performed at a rate of 90 contractions/min, each stimulus having a duration of 0.1 sec. A more prolonged stimulus (0.3 sec) was tried but proved to be associated with a larger blood flow and oxygen uptake and a larger lactate production than the shorter duration. These findings may well be due to longer periods of isometric contraction and shorter rest intervals in the rhythmic exercise performed with the longer stimulus. Moreover, exercise with the longer stimulus was usually tolerated less well and sometimes gave rise to early fatigue and discomfort, especially at a high work intensity.

The increase in voluntary work intensity to 6 and 8 kpm/min was achieved by raising the spring tension of the hand ergometer. A corresponding increase in muscle force during electrical stimulation was accomplished by increasing the strength of the stimulus. An alternative procedure would have been to increase the frequency of the square waves making up each stimulus. Preliminary experiments showed, however, that the latter procedure gave only a very modest increase in the tension developed and that "fatigue" and cessation of contractions often occurred after a few minutes, especially at stimulation frequencies of 40—60/min. This observation is in agreement with the results of Kramer, Obal and Quensel (1939) for stimulation of dog gastrocnemius and isometric work. When the developed muscle tension was raised by increasing the strength of stimulation, an increasing number of motor units became activated. Thus, this mode of raising the work intensity more closely resembles the conditions during voluntary contraction, which was considered an advantage.

During exercise induced by electrical stimulation of the median nerve one cannot avoid activating other muscles besides the superficial finger flexor and the radial portion of the deep finger flexor. Pronation of the forearm and hand occurs through the action of the pronator quadratus and pronator teres muscles. Moreover, the flexor carpi radialis and ulnaris muscles and the palmaris longus muscle are innervated by the median nerve. In the present study, movement of the hand and forearm from contraction of these muscles was prevented by performing the experiments with the forearm in a plastic casing, thereby immobilizing the radio-ulnar and carpal joints. Although there was thus no or very slight pronation or flexion of the hand, it was not possible to avoid energy expenditure from isometric contraction of the muscles concerned. This may well be one of the reasons why the electrically induced exercise is associated with a larger blood flow, oxygen uptake and lactate

production than voluntary exercise of corresponding work intensity. Several other factors may also contribute to this difference. During electrical stimulation all motor units which take part in the contraction are activated simultaneously. This may cause a larger energy expenditure in the series-coupled elastic components of muscle than if the motor units were recruited successively as occurs in voluntary contractions. Moreover, the relationship between contraction and relaxation may have been more favourable during voluntary exercise than during stimulation thus contributing to the lower mechanical efficiency during electrically induced exercise.

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Possible Functional Differentiation between the Stores from which Adrenergic Nerve Stimulation, Tyramine and Amphetamine Release Noradrenaline

By

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Abstract

OBIANWU, H O *Possible functional differentiation between the stores from which adrenergic nerve stimulation, tyramine and amphetamine release noradrenaline* Acta physiol scand 1969 *75* 92—101

Effects of inhibition of monoamine oxidase and tyrosine hydroxylase on the contraction of rat eyelid and pressor response to tyramine, amphetamine and adrenergic nerve stimulation been studied. Inhibition of MAO markedly enhanced both the peak and the duration of the eyelid to tyramine and amphetamine while only moderately augmenting the dura of pressor response to tyramine Contraction of the eyelid to nerve stimulation was not affected.

Inhibition of tyrosine hydroxylase abolished the contraction of the eyelid to amphetamine while only slightly reducing that to tyramine The contraction of the eyelid to nerve stimulation was not affected.

The results suggest that nerve stimulation tyramine and amphetamine release noradrenaline from different stores. The nature of these stores is discussed.

In recent years considerable evidence has accumulated which strongly suggests that noradrenaline stores in the sympathetic nerve terminals are not homogeneous The existence of more than one pool of noradrenaline store is now generally accepted but the functional significance of these pools is not well understood

Metabolic studies of the fate of noradrenaline released by various procedures, for example, by nerve stimulation, indirectly acting sympathomimetic amines, ganglion stimulants and reserpine-like substances have yielded useful information about the origin of the released noradrenaline

Intraneuronally released noradrenaline is largely metabolized by monoamine oxidase (MAO) while exogenously administered noradrenaline is largely metabolized by catechol-o-methyl transferase (COMT) (See Axelrod 1959, Carlsson 1965 Kopin 1964, 1966) Termination of the actions of liberated transmitter at the

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[2] A preliminary report was communicated to the joint meeting of British and Scandinavian Pharmacological Societies in Edinburgh

adrenergic nerve terminals is mainly effected by tissue reuptake process, metabolism playing a relatively minor role (See Iversen 1967) In agreement with this concept, inhibition of both MAO and COMT dose not appreciably enhance the response to adrenergic nerve stimulation In contrast to the response of nerve stimulation, inhibition of MAO greatly potentiates the response to tyramine and its α methylated analogues (Ryall 1961, Smith 1966 and Obianwu 1967 a)

According to Axelrod and Kopin (See Axelrod 1966, Kopin 1964) intraneuronal MAO is of little importance in the metabolism of noradrenaline released by the indirectly acting sympathomimetic amines This view is largely based on experiments in which urinary metabolites of noradrenaline were analysed after administration of tyramine to rats pretreated with ^{3}H noradrenaline (Kopin and Gordon 1962, 1963) However, the doses of tyramine used to induce increased urinary excretion of ^{3}H-noradrenaline and its metabolites far exceeded those which produce maximal pharmacological effects Since tyramine is a very good substrate for MAO, it is very likely that this substance in such large doses acted as a competitive inhibitor of the enzyme whose role was being investigated

In the present studies the role of MAO in the intraneuronal metabolism of noradrenaline relased by indirectly acting sympathomimetic amines or by adrenergic nerve stimulation has been investigated Attempt has also been made to differentiate the stores from which noradrenaline is released by these procedures

Methods

Male Sprague Dawley rats weighing 250—300 g were anesthetized with urethane, 1—1 2 g/kg



graph) The following procedure was adopted throughout first the responses of the eyelid to nerve stimulation at various frequencies were obtained this was followed by injection of two doses of tyramine (or α methyl tyramine) at ten minutes interval and two doses of noradrenaline The stimulation was repeated and the test drug given The whole procedure was repeated at appropriate intervals The response of the eyelid to nerve stimulation has been shown to be essentially sympathetic in nature (See Obianwu 1967 b)

Drugs used The following drugs were used l noradrenaline bitartrate d amphetamine b tartrate tyramine hydrochloride α methyl tyramine hydrobromide pargyline (Eutomyl) chlorohydrate



Braun Melsungen Germany) in order to avoid sustained sympathomimetic effects

Results

Effect of inhibition of monoamine oxidase (MAOI) on the pressor response to tyramine and response of the eyelid to tyramine and nerve stimulation

30—60 min after administration of the monoamine oxidase inhibitor (MAOI)

TABLE I Effect of MAOI pargyline, on the response of the eyelid to tyramine and pressor response to noradrenaline in anaesthetized rats

Tyramine 0 4 mg/kg (or 0 1 mg/rat) and NA 1 2 µg/kg (or 0 3 µg/rat) were injected i v at 10 min intervals before and 45—60 min after admin stration of pargyline 80 mg/kg i p Each result is the mean ± SE of number of experiments shown in brackets The eyelid was largely insensitive to the dose of NA employed

Treatment	Before pargyline		After pargyline	
	Pressor response in mm Hg	Contraction of the eyelid in mm	Pressor response in mm Hg	Contraction of the eyelid in mm
Tyramine	—	3 2 ± 0 7 (10)	—	32 5 ± 1 1 (10)
NA	43 ± 2	—	46 ± 2 p < 0 25 (10)	—

pargyline, the response of the eyelid to tyramine was markedly augmented (Table I and Fig 2) This potentiation was apparent in both the peak response and in the duration of the effect The effects of pargyline on the pressor response to tyramine and the response of the eyelid to tyramine were reamarkably different Whereas the response of the eyelid was augmented about five fold and lasted up to 40 min, the pressor response was not augmented but the duration was only prolonged for about 10—15 min

Inhibition of MAO did not significantly affect the response of the eyelid to nerve stimulation (Fig 1) The pressor response to administered noradrenaline was not influenced by pargyline (Table I) The eyelid was largely insensitive to the dose of noradrenaline employed

Effect of MAOI on the response of the eyelid to α methyl tyramine and d amphetamine

α methyl tyramine elicited more pronounced effects in both the peak and duration of the response than tyramine After MAOI administration the response of the eyelid

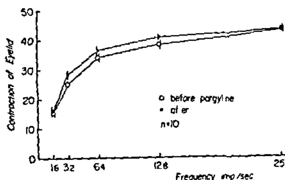


Fig 1 Effect of MAOI, pargyline on the contraction of the lower eyelid of an aesthetized rat to supramaximal preganglionic stimulation of the superior cervical sympathetic nerve Parameters of stimulation 5V 1 msec for 10 sec every 2 min ○—○ initial response ●—● response 60 min after pargyline 75 mg/kg i p — — - and p > 0 5 respectively (in increasing order of frequencies of stimulation)

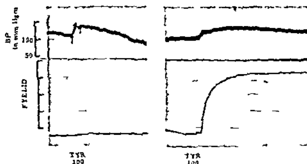


Fig 2 Effect of pargyline on the response of the eyelid and pressor response to tyramine (TYR) It shows response before (left channel) and 60 min after pargyline, 75 mg/kg i p, (right channel) The dose of tyramine is in µg/rat

to α-methyl tyramine was still augmented in 4 out of 6 animals (Fig 3) though not as much as that to tyramine However, pargyline appeared to exert some antagonistic effect on the response to α-methyl tyramine The pressor response was either very much reduced or abolished The antagonistic effect of pargyline to α-methyl tyramine was also observed on the response of the eyelid to this amine in 2 out of 6 experiments

Infusion of d amphetamine, 0 5 mg/kg elicited slight pressor response and contraction of the eyelid Amphetamine was infused in these experiments to avoid appreciable sympathomimetic effects since its influence on the response to tyramine was also being studied, the subject of another report (Obianwu 1968) 30—60 min after pargyline treatment, infusion of amphetamine elicited a marked contraction of the eyelid (Fig 4)

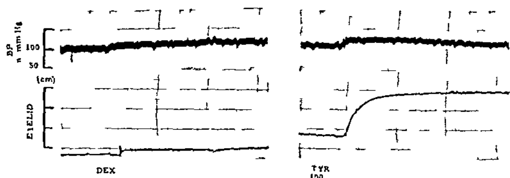
Effect of inhibition of noradrenaline synthesis on the response to tyramine and amphetamine

H 44/68, the methylester hydrochloride of dl-α-methyl-tyrosine, is an efficient inhibitor of tyrosine hydroxylase (Corrodi and Hansson 1966), an enzyme involved in the rate-limiting step of biosynthesis of noradrenaline (Nagatsu, Levitt and Udenfriend 1964) In rats pretreated with H 44/68, 250 mg/Kg, i p, 6—12 hrs before the experiment and pargyline 75 mg/kg i p 1 hr before infusion of d-amphetamine, amphetamine failed to elicit contraction of the eyelid In the same animals tyramine still elicited marked contraction of the eyelid though less than that observed in animals not treated with H 44/68 (Fig 5 and 6) H 44/68 treatment did not affect the response of the eyelid to nerve stimulation



Fig 3 Effect of pargyline on the response of the eyelid and pressor response to α methyltyramine (MTYR) in anaesthetized rat Left channel shows initial response and the right channel shows the response 60 min after pargyline, 75 mg/kg i p The dose of MTYR is in µg rat

Fig 4 Effect of pargyl ne on the response of lower eyel d to d amphetamine infus on in anaesthet zed rat a is the in t al response and b is the response 60 min after pargyl ne 75 mg/kg i p At DEX infus on of d amphetam ne (0 5 mg/kg) was commenced



he lower eyel d to infus on

in the same animal The dose of tyram ne is in µg rat

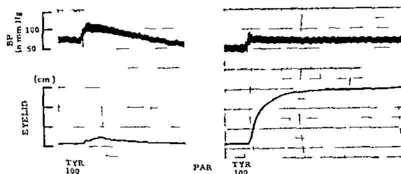


Fig 6 Effect of inhibitor on tyrosine hydroxylase on the response of lower eyelid to tyramine in anaesthetized rat Drug treatment schedule is as indicated in Fig 5 Left channel shows initial response and the right channel shows the response 60 min after pargyline (PAR) 75 mg/kg i p The dose of tyramine (TYR) is in μg/rat

Discussion

The present studies demonstrate that inhibition of MAO markedly potentiated the response of the eyelid to tyramine, α methyl tyramine and d amphetamine while not affecting that to adrenergic nerve stimulation The results also show that inhibition of noradrenaline synthesis abolished the response of the eyelid to d amphetamine while only slightl affecting that to tyramine The response to nerve stimulation was not affected

Augmentation of the response to tyramine by MAOI has been previously demonstrated Goldberg and Sjoerdsma (1959) showed that the positive chronotropic and inotropic actions of tyramine in dogs were greatly enhanced by 6 different MAO inhibitors Davey Farmer and Reinert (1963) also showed that nialamide potentiated the contraction of cat nictitating membrane to tyramine The present findings are consistent with these reports Inhibition of MAO did not alter the sensitivity of the effector organ to noradrenaline (judged by pressor response to noradrenaline) (Table I) Similar conclusion was reached by Goldberg and Sjoerdsma (1959) and Davey *et al* (1963) The effects of MAO inhibition of the contraction of the eyelid to tyramine and pressor response were remarkably different Whereas the peak of contraction of the eyelid was enhanced about five fold and the duration lasted for about 40 min, only the duration of the pressor response was prolonged for about 10—15 min Similar observation was reported by Davey *et al* (1963) Thus marked difference between the behaviour of the eyelid and the vascular system may be due to a difference in the relative importance of MAO in the inactivation of tyramine and released noradrenaline in these two situations However, in view of the complex nature of blood pressure homeostasis other explanations cannot be ruled out

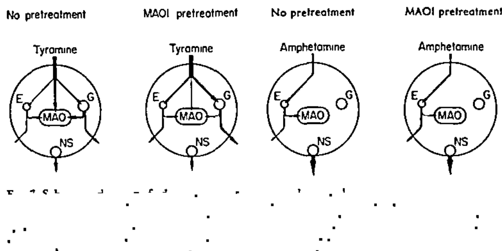
Augmentation of the response to tyramine may reflect decreased intraneuronal deamination of released noradrenaline decreased metabolism of the administered tyramine, accumulation of noradrenaline at sites from which it is released or a combination of some or all of these factors

Unlike tyramine, a methyl tyramine and d amphetamine are poor substrates for MAO Therefore, if the enhancement of the effects of tyramine after MAO inhibition was solely the result of decreased metabolism of tyramine, the effects of a methyl tyramine and d amphetamine should not be enhanced by MAO inhibitors However, pretreatment with pargyline augmented the response to a methyl tyramine and d amphetamine These observations may indicate that decreased intraneuronal deamination of released *noradrenaline rather than decreased metabolism of tyr*amine itself is more important for the potentiation of the effects of tyramine by MAO inhibitors Support for this view is provided by recent report of Smith (1966) that pargyline enhanced the effects of d amphetamine and mephentermine on isolated guinea pig atria When the noradrenaline store at the adrenergic nerve terminals has been replaced by *a* methyl noradrenaline (by pretreatment with *a* methyl dopa, Carlsson and Lindqvist 1962) the effects of tyramine on blood pressure of humans (Mc Curdy, Prange and Lopton 1964), on cat nictitating membrane (Haefely, Hurlimann and Thoenen 1966), of tyramine, d amphetamine and mephentermine on isolated guinea pig atria (Smith 1966) are enhanced In the *animals pretreated with a methyl dopa, pargyline treatment failed to enhance the* effects of tyramine, d amphetamine or mephentermine (Smith 1966)

No doubt inhibition of MAO will cause a decreased metabolism of tyramine since this amine is a very good substrate for this enzyme It is possible that only a fraction of administered tyramine is normally required for releasing maximally effective amount of noradrenaline but the observed effect is limited by intraneuronal deamination of released noradrenaline This view is not incompatible with the present findings and those of others

Increase in the amount of noradrenaline available for release within the nerve terminal after MAO inhibition is unlikely to have appreciably contributed to the enhanced response observed since after inhibition of noradrenaline synthesis the effects of tyramine were still enhanced by pargyline

Stores of noradrenaline which have a high turnover rate are more readily depleted by tyrosine hydroxylase inhibitors than those which have a low turnover rate (see Corrodi and Malmfors 1966) The observation that inhibition of this enzyme abolished the response of the eyelid to d amphetamine while only slightly affecting that to tyramine probably indicates that d amphetamine in the doses used here preferentially releases noradrenaline from a store with a high turnover rate Recently Weissman Koe and Tenen 1966) Hanson 1966) and Randrup and Munkvad (1966) showed that the central effects of d amphetamine are abolished by inhibition of tyrosine hydroxylase These reports are consistent with the present finding The nature of this store from which d amphetamine releases noradrenaline is not fully understood Reserpine treatment does not abolish the central actions of d amphetamine (See Hanson 1966 This may indicate that the central actions of d amphetamine are mediated through release of noradrenaline from a small store *which has a high turnover rate* and resistant to reserpine action d Amphetamine probably releases noradrenaline from a similar store in the peripheral nerve terminals,



though Burn and Rand (1958) reported that reserpine prevented peripheral actions of amphetamine Reserpine resistant uptake of labelled amines have been demonstrated (Anden, Carlsson and Waldeck, 1963, Stitzel and Lundborg 1967) Carlsson and Waldeck (1966) and Carlsson *et al* (1966) have shown that d amphetamine can release reserpine resistant store of noradrenaline from both central and peripheral adrenergic nerve terminals

The nature of this reserpine resistant amine store is not yet established It has been suggested to be extragranular, that is, within the nerves but outside the storage granulas (Anden *et al* 1963) But recent studies of Stitzel and Lundborg (1967) suggest that this store is particle bound

The events occurring at the adrenergic nerve terminals during nerve stimulation and after the procedures indicated in the results are presented schematically in Fig 7 To account for lack of effect of MAO on noradrenaline released by nerve stimulation, the stores from which this procedure releases noradrenaline should be located in such a way that the released noradrenaline is not metabolised by intraneuronal MAO These stores could for example be located immediately adjacent to the cell membrane of the neuron as previously suggested by De Robertis and Ferreira (1957) Since inhibition of tyrosine hydroxylase greatly antagonized the effects of ampehtamin while only slightly affecting those of tyramine, it is proposed that amphetamine preferentially releases noradrenaline from a labile store (E) which has a high turn over rate Tyramine releases noradrenaline mainly from the large stable store (G) though exerting some action on E Part of the noradrenaline released by both amines as well as part of tyramine itself are deaminated by intraneuronal MAO Inhibition of this enzyme retards deamination of tyramine as well as intraneuronally released noradrenaline, consequently causing enhanced response In accordance with this view, amphetamine in doses which produce maximal pharmacological actions has

little effect on tissue noradrenaline levels while adequate levels of tyramine can deplete tissue stores of noradrenaline by about 90 % (Neff *et al* 1965, Gutman and Weill Malherbe 1966)

Kopin and Gordon (1967, 1963) analyzed urinary metabolites of ³H noradrenaline after administration of very large doses of tyramine to rats pretreated with ³H noradrenaline and concluded that intraneuronal MAO is of little importance in the metabolism of noradrenaline released by indirectly acting sympathomimetic amines However the doses of tyramine used to produce increased urinary excretion of ³H noradrenaline and its metabolites far exceeded doses which produce maximal pharmacological effects Tyramine is a good substrate of MAO and it is likely that this amine in such large doses acted as a competitive inhibitor of the enzyme whose role was being evaluated

The present studies indicate that an appreciable proportion of noradrenaline released by tyramine and d amphetamine are metabolized by intraneuronal MAO and provide evidence which strongly suggests that the sites from which adrenergic nerve stimulation, tyramine and d amphetamine release noradrenaline may be different Recent reports of Day (1967) and others which show lack of crossed tachyphylaxis between tyramine and d amphetamine are consistent with this conclusion

The current concept of indirectly acting sympathomimetic amines (See Burn and Rand 1958 may have to be extended since it appears that tyramine like and amphetamine like sympathomimetic amines do not release noradrenaline from similar stores of noradrenaline at the adrenergic nerve terminals

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Acta physiol. scand. 1969. 75. 102—110

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Some Studies on the Mechanism by which d-Amphetamine Antagonizes Guanethidine Induced Adrenergic Neurone Blockade

By

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Abstract

OBIANWU H O *Some studies on the mechanism by which d-amphetamine antagonizes adrenergic neurone blockade induced by guanethidine* Acta physiol scand 1969 *75* 102—110

Electrical stimulation of the sympathetic trunk elicited contraction of rat lower eyelid This response was greatly reduced or abolished by guanethidine (15—25 mg/kg, s c) within 30—40 min Infusion of d amphetamine, 0 5 mg/kg greatly antagonized the blockade within 30—60 min Amphetamine similarly administered to control animals potentiated the response of the eyelid to nerve stimulation and tyramine After inhibition of MAO, amphetamine still enhanced the response to tyramine and nerve stimulation Potentiation of the response to nerve stimulation did not appear to be due to inhibition of reuptake process since this effect was still observed after protriptyline treatment, a substance which is about twenty times as active as amphetamine in this respect It is suggested that the ability of amphetamine to potentiate the response to nerve stimulation may be an important factor in its antagonism of guanethidine induced nerve blockade

A number of investigators (Laurence and Rosenheim 1960 Day 1962, Day and Rand 1963 Matsumoto and Horita 1963, Chang Costa and Brodie 1965, Brodie Chang and Costa 1965 Obianwu 1967a) have demonstrated the ability of amphetamine-like compounds to antagonize the adrenergic nerve blockade produced by guanethidine Since amphetamine can impair tissue binding of guanethidine, it was suggested that amphetamine exerts its antagonizing effects by competing with guanethidine for binding at noradrenaline storage sites (see Day and Rand 1963, Chang *et al* 1965 and Brodie *et al* 1965) However the observation that inhibition of guanethidine binding is shared by several compounds, some of which have little or no ability to antagonize guanethidine induced nerve blockade (Brodie *et al* 1965) suggests that the two processes may not be causally linked In a recent study (Obianwu, Stitzel and Lundborg 1968) it was shown that guanethidine possesses a much higher affinity for noradrenaline storage sites than amphetamine

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Considerable evidence has now been provided which show that amphetamine in doses which readily antagonize nerve blockade produced by guanethidine also potentiates the response to nerve stimulation in control animals (Ryall 1961, Day and Rand 1963, Doda, Gyorgy and Nádor 1966, Obianwu 1967a)

The ability of amphetamine to potentiate the response to nerve stimulation in control animals may be an important factor in its antagonism of neurone blockade induced by guanethidine This possibility is considered in the present studies

Methods

Male Sprague Dawley rats weighing 250—300 g, were anesthetized with urethane, 1,2 mg/kg i p, and prepared for recording of the contraction of the lower eyelid to nerve stimulation and blood pressure as previously described (Obianwu 1968) Drugs were injected into the left jugular vein in volumes of 0 1 ml and washed down with another 0 1 ml of saline Amphetamine was administered by slow infusion to avoid appreciable sympathemimetic effects Similar procedure as that described previously (Obianwu 1968) was adopted in the present studies

Results

Antagonism of guanethidine induced adrenergic nerve blockade by d amphetamine

Electrical stimulation of the sympathetic trunk elicited contraction of the eyelid Guanethidine, 15—25 mg/kg s c, almost completely blocked the response at all frequencies of stimulation employed within 15—45 min (Fig 1 and 5) At the same time the pressor response to tyramine was greatly reduced and that of administered noradrenaline was slightly potentiated These effects are consistent with previous reports of Bhagat and Shideman (1963) and Spriggs (1966) Infusion of d amphetamine, 0 5 mg/kg greatly antagonized guanethidine induced blockade of the eyelid to nerve stimulation (Fig 1) Infusion of d amphetamine also enhanced the response of the eyelid to tyramine but failed to restore pressor response to tyramine

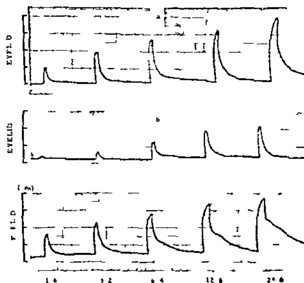


Fig 1 Contraction of lower eyelid of anesthetized rat to supramaximal preganglionic stimulation of the superior cervical sympathetic nerve Parameters of stimulation 5 V 1msec for 10 sec every two min The numbers indicate frequencies of stimulation in shocks/sec
a is the initial response b is the response 30 min after injection of guanethidine 15 mg/kg s c and c shows that 45 min after infusion of d amphetamine 0 5 mg kg the guanethidine induced nerve blockade was antagonized

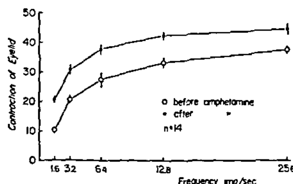


Fig 2 Effect of d amphetamine on the contraction of the lower eyelid of anesthetized rat to supramaximal preganglionic stimulation of the superior cervical sympathetic nerve Parameters of stimulation, 5 V, 1 msec for 10 sec every 2 min
o———o initial response,
•———• 45 min after infusion of d-amphetamine
Enhanced responses after amphetamine are significant The p values of control responses compared to those after amphetamine are $p<0.001$, $p<0.001$, $p<0.05$, <0.001 and $p<0.01$ respectively (in increasing order of frequency of stimulation)

Effect of d-amphetamine on the response of the eyelid to nerve stimulation and tyramine in non guanethidine treated animals

In order to elucidate the extent of a direct interaction between amphetamine and guanethidine the experiments described above were repeated in animals which had not received guanethidine Amphetamine 0.1—0.5 mg/kg was infused in a similar manner as in the rats which received guanethidine and at the appropriate intervals the responses were compared with those of control animals The results (Fig 2) show that d-amphetamine in doses which antagonize nerve blockade produced by guanethidine also markedly enhanced the response of the eyelid to nerve stimulation The response of the eyelid to tyramine was also markedly enhanced by d amphetamine infusion (Table I) while its pressor response was inhibited (Table I)

TABLE I Effect of d amphetamine on the response of the eyelid to tyramine and pressor response to tyramine and noradrenaline in anesthetized rats

Tyramine, 0.4 mg kg (or 0.1 mg/rat) and noradrenaline, 1.2 μg/kg (or 0.3 μg/rat) were injected i v at ten min intervals

d amphetamine 0.5 mg/kg, was infused slowly (20 min) by means of slow infusion apparatus (B Braun Melsungen Germany 30—60 min after commencement of the infusion, tyramine and noradrenaline injections were repeated Each result is the mean ± SE of number of experiments shown in brackets The eyelid was largely insensitive to the dose of noradrenaline employed

Treatment	Before d amphetamine infusion		After d amphetamine infusion	
	Pressor response in mm Hg	Contration of the eyelid in cm	Pressor response in mm Hg	Contraction of the eyelid in cm
Tyramine	46±4 (10)	6.4±1.1 (14)	29±6 p 0.025 (10)	26.3±2.1 (14)
NA	50±3 (10)	—	44±2 p 0.1 (10)	—

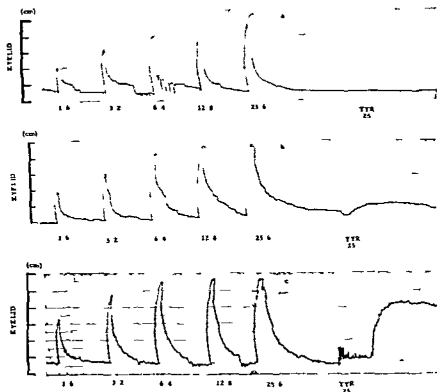


Fig 3 Contraction of the lower eyelid of anesthetized rat to supramaximal preganglionic stimulation of the superior cervical sympathetic nerve and tyramine (TYR)
a is the initial response b is the response 60 min after injection of pargyline, 75 mg/kg i p and c shows that after pargyline treatment amphetamine (45 min after its infusion), 0 5 mg/kg still potentiated the response of the eyelid to nerve stimulation and tyramine The dose of tyramine is in μg/rat and parameters of stimulation are 5 V 1 msec for 10 sec every 2 min Frequencies of stimulation in shocks/sec are as indicated.

The pressor response to injected noradrenaline was either not affected or slightly reduced (Table I) The eyelid was largely insensitive to the dose of noradrenaline employed though in a few cases where measurable response was elicited d amphetamine infusion did not affect it

Effect of d-amphetamine on the response of the eyelid to tyramine in animals pretreated with MAO inhibitor

If the augmentation of the response of the eyelid to tyramine by d amphetamine was due to inhibition of MAO this effect should not be observed after inhibition of this enzyme with a potent inhibitor Since the dose of tyramine 0 4 mg/kg employed in previous experiments elicited almost maximal contraction of the eyelid after pargyline treatment 1/4 of this dose was employed in these series of experiments After pargyline (a potent MAO inhibitor) treatment slow infusion of ampheta-

mine which in non pargyline treated animals caused only a slight contraction of the eyelid elicited a pronounced response, an effect already described in detail (Obianwu 1968) This response though starting to decline soon after cessation of infusion of amphetamine, did not quite return to the base line in most of the experiments Nevertheless the responses of the eyelid to tyramine and nerve stimulation were still enhanced (Fig 3) This may indicate that enhancement of the response of the eyelid to tyramine by amphetamine was not due to inhibition of MAO

Influence of inhibition of amine transport mechanism on the enhancement of the response of the eyelid to nerve stimulation by amphetamine

Almost all agents known to potentiate the response to adrenergic nerve stimulation owe their effect to interference with inactivation of released noradrenaline, especially by inhibition of its reuptake into adrenergic nerve terminals The doses of amphetamine employed here has only a mild inhibitory action on this uptake mechanism in vivo (Obianwu, unpublished observation, Waldeck, personal communication) Protriptyline is one of the most potent inhibitors of the uptake mechanism (Carlsson and Waldeck 1965) and it is about twenty times as active as d amphetamine in this respect (Waldeck, personal communication) Therefore if amphetamine potentiates the response to nerve stimulation by inhibition of reuptake mechanism this effect ould not be observed after protriptyline treatment Protriptyline 2 mg/kg i v enhanced the response of the eyelid to nerve stimulation and injected noradrenaline (a well known effect) (Fig 4) Infusion of d amphetamine, 0 5 mg/kg 15—30 min after protriptyline 2 mg/kg i v still potentiated the response of the eyelid to nerve stimulation This observation may indicate that inhibition of uptake mechanism is not an important factor for the potentiation of the response to nerve stimulation by amphetamine

Effect of Protriptyline on adrenergic nerve blockade produced by guanethidine

To further establish whether inhibition of uptake mechanism is a factor in the ability of amphetamine to antagonize nerve blockade produced by guanethidine or not the above experiments were repeated in animals treated with guanethidine Rats were treated with guanethidine 15—25 mg/kg s c and when the response of the eyelid to nerve stimulation was abolished protriptyline 2 mg/kg i v was given followed by amphetamine infusion 30—60 min after protriptyline Protriptyline slightly enhanced the response to nerve stimulation after guanethidine though this effect was not consistent (2 out of 4 expts) However infusion of amphetamine after protriptyline still antagonized guanethidine induced nerve blockade (Fig 5) The result suggests that inhibition of uptake mechanism is not an important factor in the ability of amphetamine to antagonize guanethidine induced nerve blockade It also indicates that amphetamine probably does not utilize the amine transport mechanism 'the membrane pump to an appreciable extent

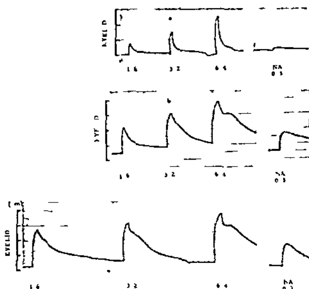


Fig 4 Potentiation of the response of the lower eyelid to supramaximal pregangliomc stimulation of the superior cervical sympathetic nerve by amphetamine after protriptyline treatment
a is the initial response b is the response 30 min after i v injection of protriptyline 2 mg/kg and c shows that after protriptyline treatment amphetamine 0 5 mg/kg (45 min after its infusion) still potentiated the response to nerve stimulation The dose of noradrenaline (NA) is in µg/rat Parameters of stimulation 5 V, 1 msec for 10 sec every 2 to 3 min Frequencies of stimulation in shocks/sec are as indicated

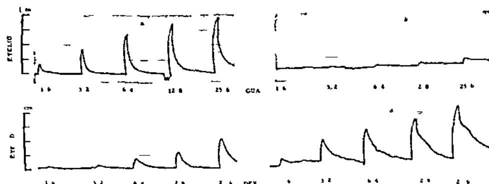


Fig 5 Failure of blockade of reuptake mechanism to prevent antagonism of guanethidine induced adrenergic nerve blockade by amphetamine
a is the initial response of the lower eyelid to supramaximal preganglionic stimulation of cervical sympathetic nerve b shows the response 45 min after s c administration of guanethidine (GUA) 20 mg/kg Between b and c protriptyline 2 mg/kg i v was administered and c was recorded 30 min after protriptyline Between c and d d amphetamine (DEX) 0 5 mg/kg was infused and d was recorded 60 min after DEX infusion Parameters of stimulation 5 V 1 msec for 10 sec every 2 min Frequencies of stimulation in shocks/sec are as indicated

Effect of inhibition of β hydroxylation on the ability of amphetamine to antagonise adrenergic nerve blockade produced by guanethidine

Evidence was recently presented which suggested that a β hydroxylated metabolite of amphetamine p hydroxynorephedrine but not amphetamine was released by adrenergic nerve stimulation after amphetamine treatment (Thoenen *et al* 1966) Inhibition of β hydroxylation with diethyldithiocarbamate (DDC) prevented release of p-hydroxynorephedrine (Thoenen *et al* 1966)

Pretreatment of animals with DDC, 500 mg/kg s c 3 hrs before the experiment did not prevent amphetamine from antagonizing nerve blockade produced by guanethidine This suggests that this antagonism is not mediated via a β hydroxylated metabolite of amphetamine (result not presented)

Discussion

The present studies show that doses of d amphetamine which readily antagonize adrenergic neurone blockade produced by guanethidine also potentiate the response to nerve stimulation in control animals This finding is consistent with those of others (Ryall 1961 Day and Rand 1963, Doda *et al* 1966, Obianwu 1967a) though Spriggs (1966) did not observe any potentiation

The mechanism of this potentiation is not known Amphetamine did not increase the sensitivity of the receptors to noradrenaline since response of the eyelid to injected noradrenaline was not potentiated Reuptake process plays an important role in the termination of actions of noradrenaline released by nerve stimulation Consequently inhibition of this process enhances the response to nerve stimulation After protriptyline a substance which is about twenty times as active as amphetamine in inhibiting reuptake process (Waldeck personal communication) amphetamine still potentiated the response of the eyelid to nerve stimulation (Fig 4) This observation indicates that the enhancement of the response to nerve stimulation by amphetamine is not due to inhibition of reuptake process It appears more likely that amphetamine facilitates release of noradrenaline from the nerve terminals In accordance with this view amphetamine enhances the response to the indirectly acting sympathomimetic amines tyramine (Table I) Ryall 1961 Eble and Rudzik 1965 and α methyltyramine (Obianwu 1967a) It has been suggested that amphetamine potentiates the response to tyramine by delaying its metabolism (Eble and Rudzik 1965 This suggestion appears unlikely since amphetamine has only a weak MAO inhibitory action in vitro though this effect does not seem to have been demonstrated in vivo In any event amphetamine still potentiates the response to tyramine in animals pretreated with MAOI pargyline (Fig 3 and enhances the response to α methyltyramine (Obianwu 1967a) a poor substrate for MAO

The suggestion that amphetamine and guanethidine compete for binding at noradrenaline storage sites (Day and Rand 1963 Chang *et al* 1965) requires that procedures which inhibit uptake and binding of noradrenaline should similarly affect binding of guanethidine and amphetamine Procedures which impair uptake and binding of noradrenaline were more effective in inhibiting binding of guanethidine than that of amphetamine For example protriptyline (or desipramine) and reserpine treatments greatly impaired tissue binding of ^{3}H guanethidine (Brodie *et al* 1965 Obianwu *et al* 1968 while exerting only a moderate inhibitory effect on that of ^{3}H amphetamine Obianwu *et al* 1968 Further sympathetic denervation greatly reduced binding of ^{3}H guanethidine but not that of ^{3}H amphetamine (Obianwu *et al* 1968)

Recently it was shown that nerve blockade induced by epsilon aminocaproic acid (EACA) is antagonized by amphetamine (Anden Henning and Obianwu 1968) but unlike guanethidine tissue binding of ^{3}H-EACA appeared not to be inhibited by amphetamine (Obianwu 1967b) The bretylium-induced nerve blockade is also antagonized by amphetamine (Lawrence and Rosenheim 1960 Wilson and Long 1960 Day 1962) even though bretylium appears to be bound at sites different from those at which guanethidine and noradrenaline are bound (Brodie *et al* 1965, Chang Chang and Su 1967) These observations suggest that the ability of amphetamine to antagonize adrenergic neurone blockade induced by these agents may not be causally related to their impaired tissue binding caused by this amine

The mechanism by which guanethidine produces adrenergic neurone blockade is not known Chang *et al* (1967) concluded that this is a consequence of rapid depletion of the particulate noradrenaline which is available for release by nerve impulse This suggestion is unlikely since after inhibition of tyrosine hydroxylase amphetamine still antagonizes nerve blockade induced by guanethidine (Obianwu 1967a) The blockade is associated with the ability of guanethidine to prevent release of noradrenaline from the nerve terminals Facilitation of release of noradrenaline is likely to antagonize this blockade Amphetamine probably achieves this effect by increasing the permeability of the cell membrane Similar suggestion based on biochemical studies was recently made by Carlsson *et al* (1966)

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Afferent and Efferent Influences on the Activity Pattern of Single Olfactory Neurons

By

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Abstract

DØVING, K B and J HYVÄRINEN *Afferent and efferent influences on the activity pattern of single olfactory neurons* Acta physiol scand 1969 75 111—123

Single unit activity was recorded from secondary olfactory neurons in the burbot (*Lota lota* [illegible] time series analyses of the impulse sequences were performed in all experimental conditions The interval histograms obtained were highly skewed The histograms of the fiber recordings were often bimodal, whereas in the microelectrode recordings several exponential histograms were found The intensity functions in two-thirds of the fiber recordings showed rhythmical time dependent oscillations in the firing probability These oscillations seemed to be of peripheral origin The activity from the microelectrode recordings usually revealed renewal processes Comparisons of the different experimental conditions showed a great variety of influences of peripheral stimuli and efferent activity on the activity patterns of the secondary neurons Most frequently the differences between different experimental conditions of one cell were smaller than the differences between cells Therefore it does not seem likely that differences in external conditions would be coded as variations in the time structure of the impulse sequence

Histological procedures have shown that there are centrifugal fibers to the olfactory bulb of vertebrates (see *e g*, Ramon y Cajal 1952 Sheldon 1912, Holmgren 1920) For a review of the mammalian olfactory system see Lohman and Lammers (1967) The first functional approach to the properties of this system was made by Kerr and Hagbarth in 1956 who observed changes in the surface potentials of one bulb while stimulating the other one Mancia *et al* (1962) observed reciprocal effects on a bulbar unit in the rabbit when they stimulated the two nostrils with the same odor

In teleosts the olfactory bulbs are connected via an efferent system that is synaptically relayed in the telencephalon (Døving and Gemne 1966) Some efferents are

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influenced by afferent activity coming from the ipsilateral bulb constituting an uncrossed efferent feedback loop analogous to the one found in the auditory system (*cf.* Fex 1965). Efferent fibers in fish have both an excitatory and inhibitory effect on the activity of the bulbar units (Døving 1966 c). About one half of the olfactory tract fibers, probably secondary neurons, are unaffected by the natural stimuli but the remaining fibers show the patterns of excitation and inhibition that are typical for the higher order neurons of other sensory systems (Nanba *et al.* 1966, Døving 1966 a).

The present experiments were performed in the hope of shedding some light on the role of the efferent system. Because of the small variations of the impulse frequency of the secondary neurons upon stimulation noted in previous studies, statistical time series analysis was applied to study the impulse sequences. This method has been used previously in the investigation of the somato sensory system (Poggio and Viernstein 1964). It would be interesting to see if this kind of analysis could give us a better understanding of the discriminatory power in the olfactory system than those procedures which have been based upon the excitatory and inhibitory types of olfactory neuron responses previously used (Døving 1965, 1966 b). Time series analysis requires long sequences of recordings with stationary conditions and since the olfactory system adapts very slowly, it may be a fruitful model organ for studying the changes in the time structure of responses to natural stimuli.

Methods

Material. The experiments were carried out on eleven burbots (*Lota lota* L.) in Oslo and Helsinki. The burbots used in Oslo were caught in Lake Malaren, Sweden, those used in Helsinki were caught in Lake Saimaa, Finland. In both places the fishes were kept in fresh water at about 12 °C. During the operative procedures they were anesthetized with tricaine methane sulphomate (MS 222, Sandoz, Basel, 1 : 20 000) and fastened to a holder for immobilization. No additional anesthesia was given. The fishes used in Helsinki were given 20—30 mg of Flaxedil to obtain better immobilization for the microelectrode penetrations. Fresh water was provided to the gills through the mouth. The skull roof was opened and mesenchymal tissue sucked away with tissue paper. The fishes used in Oslo were prepared further for the single fiber recordings by transecting the brain stem just posterior to the cerebellum to reduce movement artifacts. A small portion of the olfactory tract was cut free and single functional units obtained from the rostral end. Conventional Ag-AgCl electrodes, amplification and display systems were used. For microelectrode recording glass capillaries filled with 4 M NaCl with DC resistances of 5 to 10 megohms were applied. Filters were used to improve signal-to-noise ratio.

In what follows "*microelectrode recording*" means extracellular microelectrode recording from the olfactory bulb presumably in the vicinity of the somas of the secondary olfactory neurons. "*Single fiber recording*" means recording with single fiber technique from the afferent axons of these neurons in the olfactory tract. "*Unit recording*" means either one of these methods.

Since the main idea of the experiments was to study the afferent impulse patterns under natural stimulation with and without efferent influence, the effect of the efferent fibers was eliminated by cutting the olfactory tract posterior to the recording site. During the microelectrode recordings the tract was cooled down to about 0.5 °C. This was done by lifting the ipsilateral tract onto a silver plate that was connected to the cold side of a Peltier device. The conduction of the compound action potential of the tract was completely abolished at – °C. The cooling procedure was reversible down at least to –1 °C. By this method several units could be studied in the same preparation.

Stimuli. Three substances were used for stimulating the olfactory epithelium all at 10^{-3} M concentration. The substances were l-glutamic acid (*1*), morpholine (*2*) and taurin

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first step was to record the spontaneous activity as found in the beginning of the study of a unit (2) The olfactory epithelium was then stimulated on the side of the recording site The recording of spike activity started 10 sec after the stimulus to insure that only the steady-state activity was analysed (3) The contralateral side was stimulated with the same solution as used in step 2 (4) A control run was made at this stage to see if any changes in spontaneous activity had occurred during steps 2 and 3 (5) After abolishing the efferent activity, by cutting or cooling the olfactory tract, a new record was made of the spontaneous activity (6) In this step the ipsilateral side was stimulated with the solution used in the previous stimulations now with blocked efferent activity (7) In the cases where efferent influence was temporarily blocked by

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last digit shows the experimental step

Statistical Analysis The impulses were counted to assure a sufficient number of intervals for statistical analysis (about 500) and fed on tape for subsequent analysis The stationarity of

computer (Elliott 503)

The statistical analysis of the impulse sequences included construction of impulse *interval histograms* and calculation of the mean interval, and variance and skewness and kurtosis of the histogram *Serial correlations* with lags from 1 to 50 successive intervals were computed

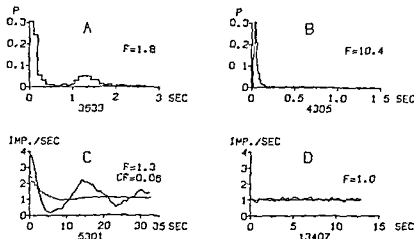
In addition to these conventional methods the probability of impulse occurrence in time was studied by computation of the *intensity function* (Cox 1965), also called expectation density (see *e g* Poggio and Viernstein 1964) The intensity function gives the conditional probability density of the occurrence of an impulse after any preceding impulse and has the dimension of impulses per second The intensity function is closely related to the autocorrelation function

Certain kinds of stochastic processes are called *renewal processes* (Cox 1962) These processes are characterized by the feature that the lengths of the intervals between the impulses are independent stochastic variables, indicating that there is no preferable sequential order in which the intervals occur The renewal processes are completely characterized by the distribution of the lengths of the intervals (interval histogram), which obviously can take any form If the order of the intervals in the sequence is shuffled at random the intensity function remains unchanged For such processes the intensity function is called *renewal density* (Cox 1965

In stochastic processes other than the renewal processes the intervals between events are dependent variables, *i e*, there is a preferable order in the sequence of intervals For these processes the random shuffling of the intervals changes the intensity function to the renewal density of the corresponding renewal process with identical interval distribution Thus shuffling of the intervals gives one possibility for investigating whether the impulse sequence is a renewal process or not Instead of shuffling the renewal density of an assumed renewal process can be estimated by successive convolutions of the interval histogram (Perkel Gerstein and Moore 1967) In the present work we estimated the renewal density through the integral equation of the renewal theory (Cox 1962 p 54) which is a more economic procedure for computer calculations.

A simple way to compare the time structure of the interval sequence with the predictions of the renewal model is to compute the intensity function from the original sequence of intervals and the corresponding renewal density from the interval histogram If both these functions follow the same time course the time structure of the interval sequence can be charactized as a renewal process If the two functions do not follow the same course there is a preferable order of intervals in the sequence

The *Poisson process* is a special renewal process with an exponential distribution of interval lengths For a Poisson process the probability of impulse occurrence is constant in time thus the intensity function and the renewal density coincide as a horizontal line



spontaneous activity of a single fiber after cutting of the olfactory tract N = 900 C Periodically oscillating intensity function (connected graph, heavy line) and the corresponding renewal density (bar graph thin line) from the spontaneous activity of a single fiber N = 500 D Intensity function and renewal density of the spontaneous activity of cell 134 showing a *Poisson process* N = 498

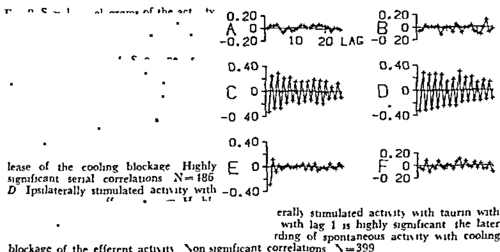
The computational methods used were based on the works of Cox (1962, 1965) and Cox and Lewis (1966) They were similar to the ones described in a previous work (Hyvärinen 1966 Thirty four single fiber recordings from 11 fibers and 29 microelectrode recordings from 12 cells were statistically analyzed All the different experimental steps were successfully analyzed for 5 units

Results

It was seldom possible to judge the changes in the structure of the impulse trains by audio-visual observation Only in some cases was there a noticeable shift from a relatively even pattern to a regularly bursting activity or vice versa No differences were visually discernible between the single-fiber and microelectrode recordings (see below)

The interval histograms were usually highly skewed with a long right hand tail (Fig 1B) Bimodal histograms were frequent in the single-fiber recordings reflecting bursting activity (Fig 1A) In these recordings no exponential histograms were found The histograms of the microelectrode recordings showed conspicuous differences, *only one bimodal and several exponential histograms were found in this* group of recordings

The intensity functions displaying the variations in the conditional probability density of the occurrence of an impulse after any preceding impulse (with the dimension of imp/sec), were periodically oscillating very slowly (0 01—1 5 c/sec) Such periodic oscillations were found only in the single-fiber recordings The period



lease of the cooling blockage Highly significant serial correlations N = 186 D Ipsilaterally stimulated activity with ... erally stimulated activity with taurin with ... with lag 1 is highly significant the later ... rding of spontaneous activity with cooling blockage of the efferent activity Non significant correlations N = 399

of the oscillations or its inverse called cyclic frequency *(CF)*, was not related to the mean discharge rate *(F)* When compared with the corresponding renewal densities the intensity functions usually revealed a periodic time dependence in the likelihood of an impulse occurrence (Fig 1C) These rhythmical oscillations in the probability of firing were as frequent with cut olfactory tract as intact olfactory tract indicating a peripheral origin of the rhythm Such time dependent processes were observed in twenty two single fiber recordings In twelve recordings no essential difference between the intensity function and the renewal density was seen indicating renewal processes

The situation differed in the microelectrode recordings which usually gave intensity functions duplicating the form of the renewal density — a characteristic feature of the renewal process In several instances, the intensity function was basically a horizontal line (Fig 1D) indicating a constant probability of occurrence of an impulse at any time after any impulse *i e*, a Poisson process On only two microelectrode recordings was a periodically oscillating intensity function found, the remaining twenty seven recordings showed a renewal process

Most of the serial correlations were nearly zero presumably because of the dominance of the variability in the few long intervals In one cell (microelectrode recording) a distinct change in the serial correlations was observed between experimental phases (Fig 2) In this cell the recording was started with the olfactory tract cooled down to +0 5° C The firing pattern was even and a flat serial correlogram was observed both in the spontaneous activity and stimulated activity (Fig 2A and 2B) When the tract was warmed up to 10° C a change to bursting activity was noted and a slight increase in frequency was also observed Simultaneously the serial correlogram became oscillating This new pattern persisted during two subsequent phases contralateral stimulation with morpoholin (Fig 2C) and

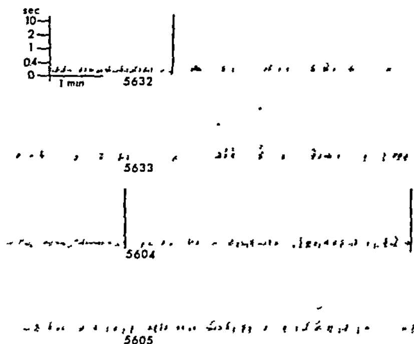


Fig 3 Photographic dot displays of intervals used for inspection of the material and judgement of stationarity of activity of fiber 56 Each dot represents one discharge the distance from the zero line to the dot corresponds to the length of the interval in a nonlinear scale The vertical bars indicate changes in the firing pattern. When no bars are marked the recordings are considered stationary and accepted for statistical analysis In other cases the recordings between the bars are used as samples In the case of this fiber changes occur in the middle of some experimental steps. All the recordings presented in the figure are with intact efferent system. *In recording 5632 the activity is recorded during ipsilateral stimulation with taurin* After about 2 min of recording a change in the firing pattern towards clustering of the spikes is seen. This pattern is then kept throughout this recording and the following recording (5633) A change occurs with the disconnection of the stimulus at the end of recording 5633 A new change occurs during recording 5604 and the new pattern is then kept throughout this recording and recording 5605

ipsilateral stimulation with taurin (Fig 2D and then gradually faded away (Fig 2E and 2F)

The occurrence of changes in the firing pattern could easily be seen in the photographic dot displays Fig 3 Sometimes changes in the firing pattern tended to be present in several successive recordings of different experimental steps The changes were never specific to any one step alone Sometimes clear changes were observed in the midst of a recording of spontaneous activity sometimes even in the midst of a stimulation period The new impulse pattern could then persist during several subsequent steps Fig 3 Such unexpected changes did not occur when the efferent activity was blocked

In general it can be said that the differences in the results of the time series analysis were more pronounced when different cells were mutually compared than when comparing the recordings of the same cell under different experimental con

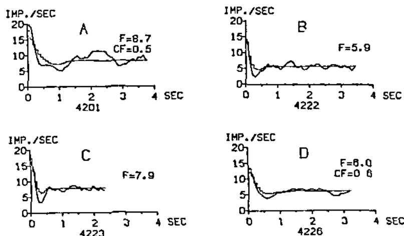


Fig 4 Intensity functions and renewal densities of the activity of fiber 42 under different experimental conditions N=500 in all graphs *A* Spontaneous activity showing periodic probability oscillations not related to renewal density *B* Ipsilateral stimulation with morpholin gives a lower discharge rate Intensity function seems to fluctuate randomly around the renewal density indicating a change to renewal process *C* Contralateral stimulation with morpholin, slight increase in discharge frequency, renewal process *D* Ipsilateral stimulation with morpholin after cutting the olfactory tract again gives periodic probability oscillation

ditions Thus the cell tended to keep its activity pattern invariant through different experimental steps, but, on the other hand the activity patterns of different cells under identical conditions often differed markedly from each other

One unit showed some unique properties and will be treated in detail below This fiber showed slight inhibition of the activity with all the stimulating solutions and was specifically tested with morpholin During the experiment, the audio-visual observation gave the impression that some change in the time series coincided with the onset of the stimulus In the time-series analysis, a periodic intensity function was noted in the spontaneous activity, initially in step *1* (Fig 4A) and in the control recording *(4)* as well as in the spontaneous *(5)* and stimulated (*6* Fig 4D) steps after cutting of the olfactory tract Both ipsilateral (*2* Fig 4B) and contralateral (*3* Fig 4C) stimulation with the efferent system intact gave a nonperiodic intensity function duplicating the renewal density, *i e* they disclosed a renewal process The histogram was bimodal in all steps Thus this particular cell showed a rhythmical time dependent activity when not influenced by efferent activity The different steps were compared by using the Student t test to show the significance of the differences in mean intervals As shown in Table 1 there was no significant difference in the mean intervals in the spontaneous recordings under identical conditions (steps *1*—*4*) Ipsilateral stimulation showed a significant inhibition when compared with spontaneous or contralaterally stimulated activity *(2—1 2—3)* When the olfactory tract was cut the spontaneous activity decreased *(1—5, 4—5)* Ipsilateral stimulation under this condition gave the same mean frequency as with intact olfactory tract but now the frequency was higher than the spontaneous activity *(5—6)*

TABLE I. The significance of differences in mean interval in the mutually compared experimental steps of recording of fiber 42. The significance levels are given as follows: *** = $p < 0.001$, ** = $p < 0.01$, 0 = $p > 0.05$. In the left column the third digit shows the stimulus used (*0* indicates spontaneous activity and *2* indicates stimulation with morpholin). The fourth digit shows the experimental step (see methods).

Recording Number	*F* imp/sec	m_1 msec	m_2	N	Significance level of difference 4201	4222	4204	4205
4201 spontaneous activity	8.7	115	56 049	546				
4222 ipsilateral stimulation	5.9	168	70 325	584	***			
4223 contralateral stimulation	7.9	127	44 190	714	0	**		
4204 spontaneous activity	7.7	130	56 332	616	0	—		
4205 spontaneous activity efferent block	4.5	220	141 680	683	***	—	***	
4226 ipsilateral stimulation efferent block	6.0	167	83 597	610	—	0	—	**

Hence the stimulus was judged as excitatory. Figure 5 shows one possibility for the connections that could bring about these results. The two secondary neurons in the bulb might be excited by the peripheral stimuli. The excitatory efferent neuron in the telencephalon is inhibited by one of these neurons and is continuously excited by some other input. When the olfactory tract is cut the excitatory influence to the olfactory neuron is abolished, thus decreasing its spontaneous frequency. The response to stimulus remains the same as previously.

The general structure of the impulse sequence as revealed by studying the histograms, serial correlations and intensity functions was usually constant throughout the different experimental steps in any one cell; therefore it was considered adequate to base the statistical tests of differences between steps on the mean of the intervals (m_1) and their variance (m_2) on which a Student t test was performed. The mean intervals varied from 96 to 1700 msec in this material. A test of signifi-

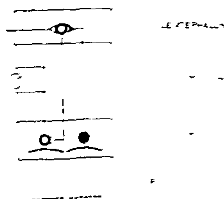


Fig. 5. A schematic diagram of the left side of the olfactory system suggesting nervous pathways for fiber 42 omitting possible interneurones. Black triangles indicate inhibitory synapses, open triangles indicate excitatory synapses. See text.

TABLE II Number of units in step comparisons arranged according to significance levels of differences in mean intervals For identification of comparisons see text

Significance level	Comparison						Total
	a	*b*	*c*	*d*	*e*	*f*	
p > 0 05	1	7	3	2	2	2	17
p < 0 05	1	3	1	—	1	1	7
p < 0 01	—	2	—	1	1	—	4
p < 0 001	3	8	3	5	5	2	26
Total	5	20	7	8	9	5	54

cant differences in the mean interval in all cells studied was performed with the following six appropriate combinations of the experimental steps (arranged in pairs)

a Spontaneous activity compared with control recordings of spontaneous activity under identifical conditions

b Spontaneous activity compared with ipsilateral stimulation (separately with intact and with blocked efferent activity)

c Spontaneous activity compared with contralateral stimulation

d Ipsilateral stimulation compared with contralateral stimulation

e Spontaneous activity with intact *efferent* activity *compared with spontaneous* activity with blocked efferent activity

f Ipsilateral stimulation with intact efferent activity compared with ipsilateral stimulation with blocked *efferent activity*

The results of these comparisons are presented in Table I for fiber 42 and in Table II for all the units studied

a Spontaneous activity The activity occurring during absence of overt stimu *lation varied considerably among the different* units The single fiber preparations that were held for sufficient time to allow for a comparison between steps *1 4*, and *7* often showed a tendency to decrease in frequencies as the experiments went along Because of the rather low frequency of impulse firing the units had to be kept for a long enough time (up to 2 hrs) to allow for a sufficient number of intervals Most probably the frequency decrease which was significant in 4 out of 5 units reflects a deterioration of the preparation

b Spontaneous activity versus ipsilateral stimulation Of the units studied twenty could be compared in steps *1—2 7—?* or *5—6* The first two comparisons were made in 10 units and in three of these there was a significant increase in activity by stimulation In 3 units there was a significant decrease in activity while 4 units did not show any sign of change in frequency

In comparing the spontaneous activity with the ipsilateral stimulation when the

efferent influence was abolished we found a significant increase in 3 units, decrease in 4 units, and an insignificant change in activity in 3 units. The fiber 42, which was discussed at some length above, was peculiar in that it was inhibited by stimulation with the efferents intact, but excited by the same stimulus after cutting the tract.

c. Spontaneous activity versus contralateral stimulation. In seven units the spontaneous activity could be compared to the activity obtained during stimulation of the contralateral side. All units showed a decrease in frequency, but it was only significant in 4 of these units.

d. Ipsilateral versus contralateral stimulation. From comparisons of the effect upon stimulation of either of the two sides, it is apparent that contralateral stimulation both facilitated and inhibited the impulse discharge from the bulbar units in respect to stimulation of the ipsilateral side. There was a significantly higher frequency when stimulating the opposite side than when stimulating the ipsilateral side in 3 units, significantly lower frequency in 3 units, and nonsignificant variations in 2 other units.

The efferents can excite or inhibit the activity of the bulbar neurons, the facilitation of impulse discharge can therefore be a release of inhibitory activity or a direct increase in activity of the excitatory input. By judging the spontaneous activity before and after abolishing efferent activity, one can obtain a picture of the general effect of the efferent fibers on the bulbar units. In most cases, when we compared the ipsi and contralateral stimulation, there were no differences. In 2 units where the efferents had inhibitory influence, contralateral stimulation gave higher frequency than ipsilateral stimulation in 1 unit and lower frequency in another. In another unit where the efferents had an excitatory influence, the frequency evoked by contralateral stimulation was higher than by ipsilateral stimulation.

e. Spontaneous activity with and without efferent influence. In two units there was a significant increase in the discharge rate after abolishing efferent influence. In 5 units there was a significant decrease in activity after cutting or cooling the olfactory tract. These results are in accordance with the previous results on the efferent system (Døving 1966a), showing that the efferent influence has both excitatory and inhibitory effect on the secondary neurons of the bulb.

f. Ipsilateral stimulation with and without efferent influence. In 3 units the frequency was significantly higher with the efferent system intact than with blocked efferent influence. In 2 units there were no differences.

Discussion

A striking phenomenon in the recordings is the general stability of the response pattern in most cells throughout the different experimental steps. The pattern is also usually preserved when changes in mean frequency occur with the application of odorous stimuli. On the other hand, the activity patterns of different cells in identical conditions often differed markedly from each other and these differences were usually preserved during all the experimental phases. On the basis of the above

findings it seems likely that differences in external conditions, for example differences in the quality of the stimulants, are not coded as variations in the time structure of the impulse sequence sent toward the brain

In some units a basic stable activity pattern was recorded, but a sudden change to another pattern could occur (Fig 3) These changes seemed unrelated to the experimental variables The new pattern was preserved for a long time after application of the stimulus and during other subsequent procedures Such variations have also been reported by Nanba *et al* (1966) As stressed by these authors, it seems necessary to compare the effect of stimulation on the activity pattern with frequent control recordings of the spontaneous activity

In this study we found it difficult to keep these slowly discharging units for a sufficient time to allow a complete statistical analysis of all the experimental steps Most frequently the units were lost before the experimental procedure was finished The small size of the fibers in the olfactory tract of fish may contribute to this instability (Døving and Gemne 1965, Døving 1967) Also it is difficult to use a closed chamber for microelectrodes in the fish skull The deterioration could sometimes be explained by the fact that some of the recurrent blood flow leaves the olfactory bulb via the veins in the olfactory tract, and in the cutting or lifting of the tract, which was necessary for cooling, the blood flow would decrease

A reliable comparison of differences between experimental steps was rendered impossible because the spontaneous activity, when repeatedly tested under identical conditions, showed significant variability and sudden changes were sometimes noted in the midst of some invariant experimental steps These difficulties in the interpretation of the results call for complete standardization of the experimental conditions in order to compare the different experimental steps It seems mandatory to base firm conclusions on a larger amount of recordings than those made for this study

In about two-thirds of the single fiber recordings a rhythmical oscillation in the probability of firing in the secondary neurons was found This rhythm was not abolished by cutting or cooling the olfactory tract Therefore it must be considered to be of peripheral origin. However in some units peripheral stimulation with the efferent system intact seemed to be able to prevent the formation of this rhythm because stimulation with the efferent system blocked did not prevent the rhythm (Fig 4)

The results of the present studies confirm earlier findings with electrical stimulation (Døving 1966c) showing that the efferent activity has an influence upon the response patterns of the secondary neurons in the olfactory system There were significant differences in the impulse frequencies as well as the time structure of the impulse sequences between the different steps The various stimuli and treatments of the olfactory system influenced the activity of the units in all possible ways The multitude of influences which was uncovered by the experimental treatment makes it very difficult to get any clear cut picture of the function of the efferent system under natural stimulation

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Formation of Extracellular Adenosine Triphosphate by Tumour Cells

By

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Abstract

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The formation of extracellular ATP by Ehrlich ascites tumour cells and HeLa cells was studied

during incubation was necessary for the main part of (^{32}P)ATP to be formed It is therefore concluded that most of the (^{32}P)ATP present in the extracellular medium was formed by enzymes located on the surface of the tumour cells

Previous work in this laboratory has shown that intact human erythrocytes (Ronquist 1968) are capable of forming extracellular, labelled ATP when incubated with all substrates and cofactors (including (^{32}P)orthophosphate) of the glyceraldehyde 3-phosphate dehydrogenase (D glyceraldehyde-3 phosphate NAD$^+$ oxidoreductase (phosphorylating), E C 1 2 1 12) and phosphoglycerate kinase (ATP D 3 phosphoglycerate 1-phosphotransferase, E C 2 7 2 3) reactions

These studies have now been extended to other cells Ehrlich and HeLa tumour cells, cultivated in tissue culture, were suitable for such studies because of their relative uniformity and of the relative ease of making homogeneous suspensions The tumour cells were incubated under isotonic conditions and the intra and extracellular incorporation of (^{32}P)orthophosphate into ADP and ATP was studied

Material and methods

Cells and media

A hyperdiploid strain of Ehrlich mouse-ascites tumour cells grown in mice stem ABM/dba was immediately brought to 4° C after removal from the peritoneal cavity (A generous gift from Professor

G KLEIN Karolinska Institutet Stockholm) HeLa cells (by courtesy of Dr L Philipson Department of Medical Microbiology Uppsala) were grown as previously described (Philipson 1967) The preparative and incubation procedure which was the same for both Ehrlich and HeLa

Prewarming and incubation of tumour cells

The volume of the cell suspensions used in each incubation was 9 5 ml The experiments with

experiments was 10 ml The $^{32}P_i$ (Radiochemical Centre Amersham Bucks England) was purified before use as previously described (Ronquist 1967) The incubations were carried out in polyethylene tubes with gentle bubbling of nitrogen gas into the medium The reactions were terminated at the end of the incubation periods in two ways

In the experiments accounted for in Table I the reactions were stopped by adding 3 0 ml of 3 N perchloric acid to the cell suspensions followed by 10 ml of ice cold distilled water The acid extract from the precipitated cells was filtered before neutralization with potassium hydroxide

In the experiments listed in Table II the incubated cell suspensions were centrifuged for 3 min at 700 × *g* The supernatant (extracellular medium) which separated easily from the

acid extract from the precipitated cells was filtered before neutralization with potassium hydroxide

with potassium hydroxide

All neutralized perchloric acid extracts were kept overnight at 4 C before filtration The labelled products were separated by ion-exchange chromatography and analyzed as described previously (Ronquist 1968) Orthophosphate was determined by the method of Martin and Doty (1949) Total phosphorus was assayed by the same method after digestion of the samples according to Grunbaum Schaffer and Kirk (1952)

Results and discussion

In Table I a comparison is made between the total formation of (^{32}P)ATP by cells incubated in the complete and incomplete systems At 1 minute's incubation the amount of (^{32}P)ATP formed in the complete system was about 8 times that in the incomplete system

Provided that ADP NAD and glyceraldehyde 3 phosphoric acid do not penetrate the cell membrane the difference could be due to enzymes located on the surface of the tumour cells It was therefore necessary to exclude any diffusion of

TABLE 1 Total (^{32}P)ADP and (^{32}P)ATP formation on incubation with complete and incomplete systems.

	30 sec		60 sec	
	(^{32}P)ADP	(^{32}P)ATP	(^{32}P)ADP	(^{32}P)ATP
Complete system				
Ehrlich cells	0.007 (1.90)	0.050 (0.75)	0.019 (1.87)	0.098 (0.85)
HeLa cells	0.011 (2.20)	0.069 (0.85)	0.027 (2.13)	0.130 (0.92)
Incomplete system				
Ehrlich cells	0.001 (0.18)	0.005 (0.52)	0.005 (0.14)	0.013 (0.54)
HeLa cells	0.002 (0.20)	0.008 (0.56)	0.007 (0.21)	0.016 (0.55)

The results given are the incorporation of (^{32}P)orthophosphate (in μmoles) into ADP and ATP per 100 mg dry weight of Ehrlich ascites tumour cells and HeLa cells. Figures in brackets denote the total amount of ADP and ATP recovered after the incubation period (μmoles per 100 mg dry weight of tumour cells).

intracellular enzymes, as well as of intracellular metabolites into the extracellular medium. Furthermore, the distribution of (^{32}P)ATP between the intra- and extracellular spaces on incubation with the complete system had to be determined. Data given in Table 2 confirm that all of the (^{32}P)ATP, the formation of which can be attributed to the added ADP, NAD$^+$ and glyceraldehyde-3-phosphoric acid, was recovered in the extracellular medium.

At 1 minute's incubation the specific radioactivity of extracellular (^{32}P)ATP was about 4 times lower than that of extracellular (^{32}P)orthophosphate. Since the data on the incomplete system show that no measurable leakage of intracellular (^{32}P)ATP occurred, the aforementioned difference in specific radioactivity may be explained by the presence of adenylate kinase (ATP: AMP phosphotransferase, E.C. 2.7.4.3) on the cell surface, or soluble in the extracellular medium. The presence of adenylate kinase activity also accords with the observation that extracellular AMP was formed in stoichiometric amounts to extracellular ATP. The labelling of ADP may also have been due to the adenylate kinase activity.

The incorporation of (^{32}P)orthophosphate into intracellular ADP and ATP was low compared with the corresponding incorporation into extracellular ADP and ATP by tumour cells incubated with the complete system (see Table 2). It must, however, be borne in mind that there is a relatively slow penetration of (^{32}P)orthophosphate into the Ehrlich and HeLa cells, since the intra- and extracellular distribution of (^{32}P)orthophosphate has not come into equilibrium during 1 minute's incubation. The specific radioactivity of the extracellular orthophosphate at 1 minute's incubation was 21.7 $\times$ 10^{5} cpm/μmole for Ehrlich ascites tumour cells, and 19.1 $\times$ 10^{5} cpm/μmole for HeLa cells, respectively, while the intracellular specific radioactivity was 2.8 $\times$ 10^{5} and 2.3 $\times$ 10^{5} cpm/μmole, respectively.

TABLE II Extra and intracellular distribution of (^{32}P)orthophosphate incorporated into nucleotides of tumour cells incubated with the complete and incomplete system

	30 sec				60 sec			
	Extracellular medium		Intracellular medium		Extracellular medium		Intracellular medium	
	(^{32}P)ADP	(^{32}P)ATP	(^{32}P)ADP	(^{32}P)ATP	(^{32}P)ADP	(^{32}P)ATP	(^{32}P)ADP	(^{32}P)ATP
Complete system								
Ehrlich cells	0 062	0 080	0 002	0 007	0 130	0 175	0 005	0 014
	(1 54)	(0 69)	(0 29)	(0 60)	(1 58)	(0 68)	(0 21)	(0 56)
HeLa cells	0 070	0 124	0 003	0 008	0 163	0 236	0 004	0 017
	(1 63)	(0 81)	(0 32)	(0 63)	(1 53)	(0 83)	(0 28)	(0 57)
Incomplete system								
Ehrlich cells	0 001	0 003	0 002	0 007	0 002	0 003	0 005	0 014
	(0)	(0)	(0 21)	(0 53)	(0)	(0)	(0 16)	(0 52)
HeLa cells	0 002	0 003	0 003	0 007	0 002	0 004	0 006	0 015
	(0)	(0)	(0.24)	(0 60)	(0)	(0)	(0 26)	(0 60)

The results given are the incorporation of (^{32}P)orthophosphate into ADP and ATP expressed in µmoles per 100 mg dry weight of tumour cells Figures in brackets are the total amount of ADP and ATP recovered after the incubation period (µmoles per 100 mg dry weight of tumour cells)

No measurable difference was found between the (^{32}P)orthophosphate penetration into tumour cells incubated in the complete and the incomplete system Thus, the intracellular incorporation of (^{32}P)orthophosphate into ADP and ATP was independent of extracellular conditions The results also confirm that the (^{32}P)ATP found in the extracellular medium of tumour cells incubated with the complete system was not of intracellular origin

TABLE III Formation of (^{32}P)ADP and (^{32}P)ATP by cell free supernatant from tumour cells

	30 sec		60 sec	
	(^{32}P)ADP	(^{32}P)ATP	(^{32}P)ADP	(^{32}P)ATP
Supernatant (Ehrlich cells)	0 002 (1 24)	0 004 (0 55)	0 005 (1 12)	0 00[illegible]
Supernatant (HeLa cells)	0 002 (1 31)	0 005 (0 68)	0 005 (1 23)	0 012 (0 80)

Supernatants obtained after centrifugation of pre warmed tumour cell suspensions at 37°C followed by incubation with the complete system at the same temperature The results are given in µmoles of (^{32}P)orthophosphate incorporated into ADP and ATP per 100 mg dry weight of tumour cells Figures in brackets are the total amount of ADP and ATP recovered after the incubation period (µmoles per 100 mg dry weight of tumour cells)

A cell free supernatant from the pre warmed tumour cell suspension, serving as a control of any enzyme leakage from the tumour cells, was also incubated with the complete system Table 3 shows that only relatively small amounts of (^{32}P)ortho phosphate were incorporated into ADP and ATP *It is not possible to determine* by the present methods whether the enzymes responsible for this incorporation had leaked out from the cells or had been eluted from the cell membranes In any case, the presence of intact tumour cells in the incubation medium is necessary for the formation of the main part of the extracellular (^{32}P)ADP and (^{32}P)ATP on incubation with the complete system (see Table I and II) These findings therefore strongly support the view that glyceraldehyde-3 phosphate dehydrogenase and phosphoglycerate kinase are located on the surface of the tumour cells

It is also seen from the figures in Table 3 that the conversion of ADP into ATP by the cell free supernatant is of the same order of magnitude as that in the extra cellular medium of cells incubated with the complete system (Table II) The adenylate kinase thus seems to be easily eluted from the tumour cells This is in agreement with the work of Manery, Riordan and Boegman (1967) on muscle cells, as well as with an earlier investigation on *human* erythrocytes *(Ronquist 1968)*

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Studies on the Effect of Adrenergic Blocking Drugs on Catecholamine-Induced Platelet Aggregation and Uptake of Noradrenaline and 5-Hydroxytryptamine

By

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Abstract

Bygdeman, S and Ø Johnsen *Studies on the effect of adrenergic blocking drugs on catecholamine-induced platelet aggregation and uptake of noradrenaline and 5-hydroxytryptamine* Acta physiol scand 1969 75 129—138

Adrenaline (A) and noradrenaline (NA) were both found to induce platelet aggregation in [illegible]

blocking agents and their effect on catecholamine induced aggregation indicating that uptake of A or NA was not a necessary step in catecholamine mediated platelet aggregation

Adhesion and aggregation of platelets at the site of a lesion of the intima constitute the first two steps in thrombus formation (*cf* Marcus and Zucker 1965) Aggregation of platelets can be induced *in vitro* in citrated platelet rich plasma (CPRP) by adenosine diphosphate (ADP) and by a number of other substances including thrombin, adrenaline (A) noradrenaline (NA) and 5-hydroxytryptamine (5 HT) (Gaarder *et al* 1961 Shermer *et al* 1961 O'Brien 1963 1964 Clayton and Cross 1963 Haslam 1964 Mitchell and Sharp 1964 Schwartz and Ardlie 1967 Jackson *et al* 1967 Mills and Roberts 1967) The aggregating response to these substances is in most cases mediated through a release of ADP from the platelets (Haslam 1964 1967) The concentration of catecholamines used to induce aggregation in human CPRP has been in the order of 10^{-6} M A being equally or somewhat more

effective than NA (Mitchell and Sharp 1964, O Brien 1963, Schwartz and Ardlie 1967) Markedly lower concentrations of especially A can potentiate ADP induced aggregation (Ardlie Glew and Schwartz 1966, Ardlie and Schwartz 1967) It is therefore conceivable that catecholamines can play a role in thrombus formation *in vivo* at least under stress conditions with an increased blood catecholamine concentration. This hypothesis is further supported by the observation that infusion of A to humans or rabbits can increase the number of adhesive platelets (Vaughan Jones Ingram and Mc Clure 1963) and the tendency to thrombus formation (Rowsell *et al* 1966) No aggregating response has been observed with isoprenaline (Clayton and Cross 1963) The effect of catecholamines can be blocked by phentolamine (O'Brien 1963) but also by dichloro-isopropylnoradrenaline (Clayton and Cross 1963) The results presented have generally been interpreted to indicate, although conclusive evidence is still lacking, that catecholamine induced aggregation is mediated through an α-receptor mechanism

Human blood platelets contain considerable amounts of 5-HT but almost no A and NA although blood platelets from most species have the ability to take up these substances actively under suitable incubation conditions (Hardisty and Stacey 1955, Zucker and Borelli 1955 Born Hornykiewicz and Stafford 1958, Born and Gillison 1959 Sano *et al* 1959 Hughes and Brodie 1959, Weissbach and Redfield 1960 Stacey 1961)

The aim of the present study has been to further evaluate the platelet aggregating effect of catecholamines The relative effectiveness of a series of α- and β-receptor blocking agents on catecholamine induced platelet aggregation has been determined and the relation between uptake and aggregating effect has been evaluated A preliminary account of the results has been presented (Bygdeman 1968)

Methods

Healthy male volunteers between 20 and 25 years of age were used in the study Blood samples were taken from ante-cubital veins through wide bore stainless steel needles The blood was collected in glass tubes containing 1/10 volume of 3.13 per cent trisodium citrate dihydrate or 1/15 volume of 0.077 M EDTA. To obtain platelet rich plasma the whole blood was centrifuged 30 min after collection at $290 \times g$ and room temperature for 15 min All glass and metal surfaces in contact with blood or plasma were siliconized

Platelet aggregation was determined in CPRP with a modification of the turbidometric method originally described by Born 1962 A double beam spectrophotometer (Beckman DB) was used Both the reference and the sample cell of the spectrophotometer were filled with 1.8 ml of CPRP The α and β receptor blocking agent was then added in a volume of 0.1 ml to each cuvette Under these circumstances when the optical densities (read at 6000 Å) of the sample and reference cells are identical the instrument indicates a transmission of 100 per cent In the next step 0.1 ml of an ADP or catecholamine solution was added to the reference cell and 0.1 ml normal saline to the other cell The degree of aggregation induced could be followed by recording the change in optical density With this method changes in optical density in both the sample and reference cells were automatically compensated for Stirring in the cells was achieved with a small synchronous motor rotating two threaded teflon rods with a speed of 1000 rpm The stirring device was built into the sample compartment The change in optical density was recorded on a Beckman potentiometric recorder using a paper speed of 2.5 cm/15 sec The slope of the curve at the steepest part was determined and expressed as change in optical density per 15 sec (Eliasson and Bygdeman to be publ)

The uptake of ^{3}H—NA and ^{14}C-5 HT in the platelets was studied in EDTA and citrated PRP Tracer amounts of the amines were added 10^{-8} M The uptake of radioactively

labelled NA and 5 HT was determined at 5°, 20°, and 37° C for up to 180 min At the end of the incubation period the plasma was centrifuged at 4° C for 15 min at 700×g The supernatant was decanted and drained from the pellet of sedimented platelets by turning the

ried inside with filter paper to just above the pellet. The
r pipette in a volume equal to the plasma of a medium
THAM HCl buffert pH 7 4 and 0 077 M EDTA mixed

in the proportion 90—8—2 by volume (Haslam 1964) The suspension was centrifuged at 4° C for 15 min at 700×g The supernatant was decanted and the platelet pellet resuspended in the salt solution as described above Trichloracetic acid (50 %) solution) was then added in an amount necessary to obtain a final concentration of 5 %, to extract the amines After centrifugation aliquotes of the supernatant were measured in a Packard liquid scintillation counter

counting the number of platelets in the supernatants and following resuspension with an electronic particle counter (Celloscope 207, Ljungberg Ltd Sweden) The number of platelets remaining after the last resuspension varied in different series of experiments between 70 and 90 per cent However, in parallel experiments using the same plasma no significant differences in platelet counts could be found between different samples

The effect of blocking agents on uptake of ^{3}H NA and ^{14}C-5 HT was studied in both citrated and EDTA PRP Incubation temperature was in these experiments 37° C and incubation time 90 min

The following drugs were used

Phenoxybenzamine (Dibenyline®, Smith Kline and French), phentolamine (Regitin®, CIBA) dibenamine dichloroisoproterenol (Lilly and Co) pronethalol (Alderlin® ICI), propranolol (Inderal®, ICI), MJ 1998 and 1999 (Mead Johnson Research Center Evansville)

Results

Platelet aggregation

Both A and NA were found to induce platelet aggregation in citrated platelet rich plasma (Fig 1) No aggregation response was observed with the same concentrations of isoproterenol (ISO) The relative potencies were found to be about 7—8 for A for NA 1 and for ISO 0 The aggregation response to catecholamines were stereospecific, the l form of NA being more than 30 times as effective as the d form (Fig 2)

Catecholamine induced aggregation could be blocked by low concentrations of phentolamine as shown in Fig 3 and 4 while it was necessary to markedly increase the concentration of the blocker to inhibit the effect of ADP (Fig 5) Beta receptor blocking agents could also inhibit the aggregation response to A and NA although only in high concentrations (Fig 6) The results obtained with the receptor blockers tested are summarized in Table I

Uptake of ^{3}H NA and ^{14}C 5 HT

Platelets incubated with ^{3}H NA or ^{14}C-5 HT in platelet rich plasma take up the amines The uptake is temperature dependent and the uptake curves are shown in Fig 7 and 8 In order to establish to what extent inhibition of uptake was the main

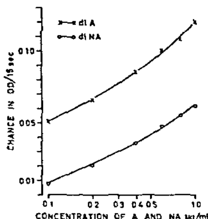


Fig 1

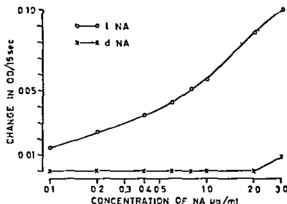


Fig 2

Fig 1 Platelet aggregation in human citrated platelet rich plasma following the addition of different doses of adrenaline (A) and noradrenaline (NA)

Fig 2 Platelet aggregation in human citrated platelet rich plasma following the addition of l and d noradrenaline (NA)

cause of the effect of α and β receptor blocking agents on catecholamine-induced aggregation the effect of a series of different blockers on uptake of ^{3}H-NA was determined To test the specificity of an inhibition of uptake of ^{3}H-NA by the blockers similar studies were carried out with ^{14}C-5 HT and the same blocking drugs The results obtained are summarized in Table II—V As can be seen in the tables the uptake of ^{3}H NA in the platelets can be blocked by both α- and β-receptor blocking agents No difference in the degree blockade was observed between experiments carried out in citrated or EDTA platelet rich plasma although

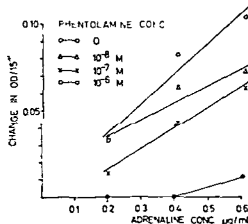


Fig 3

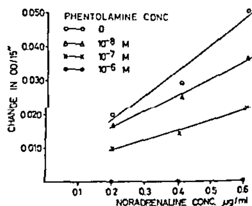


Fig 3

Fig 3 Effect of different concentrations of phentolamine on adrenaline induced platelet aggregation in human citrated platelet rich plasma

Fig 4 Effect of different concentrations of phentolamine on noradrenaline induced platelet aggregation in human citrated platelet rich plasma

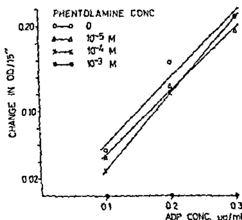


Fig 5

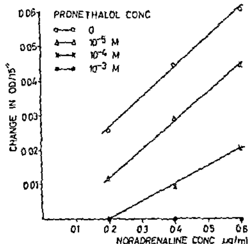


Fig 6

Fig 5 Effect of different concentrations of phentolamine on ADP induced aggregation in citrated platelet rich human plasma

Fig 6 Effect of pronethalol in different concentrations on noradrenaline induced platelet aggregation in human citrated platelet rich plasma

the total amount of amine taken up was bigger in the citrated plasma There was no obvious relationship between the concentrations which counteracted catecholamine-induced aggregation and those necessary for inhibition of uptake For instance phentolamine in a concentration of 10^{-6} could completely inhibit A and NA induced aggregation while a concentration of 10^{-3} is necessary to completely inhibit platelet uptake of NA

Discussion

The results obtained confirm the platelet aggregating effect of catecholamines earlier reported The relative responsiveness to a selected series of catecholamines strongly indicated that the response was mediated by an α-receptor mechanism according to the classical concept (Ahlquist 1948, Furchgott 1967) This conclusion was further supported by the stereospecificity of the response the natural l form being more than 30 times as effective as the d form

The observation that high concentrations of β receptor blocking agents could inhibit the aggregation following addition of A and NA does not inv[illegible]e this conclusion since at this concentration level an unspecific effect of the[illegible]s was observed as indicated by the inhibition of ADP induced aggregation[illegible]ing t the studies reported by McMillan (1966) and Mills and Roberts (1[illegible]nd N in high doses can induce a diphasic aggregation response, the se[illegible]e m likely being due to a release of ADP [illegible] presented (Haslam 1967) that also

TABLE 1. Effect of different α- or β-receptor blocking agents on A, NA and ADP induced aggregation in citrated platelet rich human plasma. The figures indicate aggregation response in % of control

α- or β-receptor	molar conc	Concentration (in μg/ml) of A			NA			ADP		
		0.2	0.4	0.6	0.2	0.4	0.6	0.1	0.2	0.3
phentolamine	10[illegible]				57	58	71			
	10[illegible]	61	71	73	38	24	43			
	10[illegible]	5	6	11	0	0	0			
	10[illegible]							101	94	99
	10[illegible]							28	73	93
	10[illegible]							0	0	0
phenoxy	10[illegible]				67	82	92			
benzamine	10[illegible]	109	111	90	59	74	73	113	111	101
	10[illegible]	7	14	13	0	0	0	79	88	87
	10[illegible]	0	0	0				21	42	59
dibenamine	10[illegible]					100	109			
	10[illegible]	104	101	93		105	104		105	104
	10[illegible]	41	41	45	36	41	41	88	100	98
	10[illegible]	0	0	0	0	0	0	23	37	54
pronethalol	10[illegible]		99	115	67	72	65	101	105	105
	10[illegible]	79	77	83	7	37	44	85	96	100
	10[illegible]	0	0	0	0	0	0	0	0	8
MJ 1999	10[illegible]	117	117	178	98	104	87			
	10[illegible]	105	88	170	81	81	97	107	121	104
	10[illegible]	78	55	85	35	53	54	94		10
		One experiment						(One experiment		
propranolol	10[illegible]		105	100	116	115	105	107	92	9
	10[illegible]	56	55	66	9	27	27	84	107	119
	10[illegible]	0	0	0	0	0	0	0	0	0

which has been investigated in the present study is mediated through a release of ADP from the platelets. If this is the case the inhibition of A and NA induced aggregation by β-blockers could be explained by this effect on ADP induced aggregation without assuming any interference with the α receptor mechanism. It is in this connection interesting to note that MJ 1999 which only moderately inhibited the effect of A and NA did not interfere with ADP induced aggregation. The concept of an α receptor mechanism involved in the aggregating effect of A and NA was further supported by the inhibition observed with low concentrations of phentol-

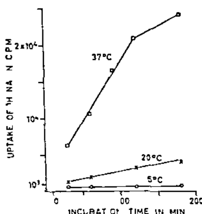


Fig 7

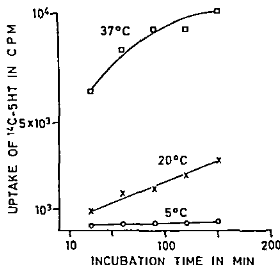


Fig 8

Fig 7 Platelet uptake of ^{3}H NA at different temperatures in citrated platelet human rich plasma

Fig 8 Platelet uptake of ^{14}C 5 HT at different temperatures in human citrated platelet rich plasma

amine, which is in accordance with earlier reports (O Brien 1963, Mills and Roberts 1967) In contrast to the results of Mills and Roberts (1967) dibenamine and dibenzyline could both inhibit A and NA induced aggregation though only in high concentrations It is important to note however that in contrast to β receptor blockers all α receptor blocking agents inhibited A and NA induced aggregation in a concentration 10 to 1000 times lower than that necessary to block the effect of ADP

Certain α and β receptor blocking agents have been shown to block the uptake of NA into tissues (Axelrod Hertting and Potter 1962 Farrant Harvey and Pen

TABLE II Effect of α receptor blocking agents on uptake of H^3 NA in human platelets (EDTA platelet rich plasma)

Uptake of H^3 NA in per cent of control						
Conc M	phentol amine	phenoxy benzamine	prone thalol	dichloro isoprot	MJ 1999	MJ 1998
10^{-4}	27	54	28	35	84	86
10^{-5}	44	89	44	43	92	89
10^{-6}	81	94	81	75	94	89
10^{-7}	95	95	95	93	96	94

TABLE III. Effect of α- and β-receptor blocking agents on uptake of H³-NA in human platelets (citrated platelet rich plasma)

Uptake of H³-NA in per cent of control

Conc. M	phentolamine	dibenamine	phenoxybenzamine	dichloroisoprot.	pronethalol	propranolol dextro	propranolol laevo	MJ 1999
10^{-3}	8	[illegible]2	32	18	25	13	15	60
10^{-4}	28	81	67	35	26	31	31	88
10^{-5}	54	92	85	48	34	49	49	80
10^{-6}	83	100	88	107	76	99	92	91
10^{-7}	88	102	92	91	84	98	89	87

TABLE IV. Effect of α- and β-receptor blocking agents on uptake of 5-HT in human blood platelets (EDTA platelet rich plasma)

Uptake of 5-HT in per cent of control

Conc. M	phentolamine	phenoxybenzamine	dibenamine	pronethalol	dichloroisoprot	propranolol	MJ 1999
10^{-3}	24	24	47	6	3	3	102
10^{-4}	[illegible]	89	91	17	8	29	102
10^{-5}	93	89	96	45	49	68	103
10^{-6}	—	—	102	83	94	77	100
10^{-7}	95		—	[illegible]	[illegible]	90	102

TABLE V. Effect of α- and β-receptor blocking agents on uptake of 5-HT in human blood platelets (citrated platelet rich plasma)

Uptake of 5-HT in per cent of control

Conc. M	phentolamine	phenoxybenzamine	dibenamine	pronethalol	dichloroisoprot	propranolol	MJ 1999
10^{-3}	39	44	71	16	2	18	93
10^{-4}	95	[illegible]	99	61	37	80	101
10^{-5}	94	[illegible]	10[illegible]	89	89	92	101
10^{-6}	[illegible]	[illegible]	104	92	97	99	102
10^{-7}	97	100	104	99	104	100	103

nefather 1964, Lindmar and Muscholl 1964, Iversen 1965) In a systematic study it has been demonstrated that most of the α and β-blockers inhibit the uptake of NA in isolated adrenergic nerve granules (Euler and Lishajko 1966, 1968) The results obtained in the present study demonstrate that α- and β receptor blocking agents could also inhibit the uptake of NA in the platelets It is interesting to note that the concentrations necessary to block the uptake of NA into platelets were well correlated to those inhibiting granular uptake This agreement was also well illustrated by the action of the β blocking drugs MJ 1998 and 1999, which had only a weak action on the uptake of NA in nerve granules as well as on the NA uptake in platelets in the present study It was also observed that l- and d propranolol, which had similar inhibitory action on the NA uptake in nerve granules, showed no difference in action on the NA uptake in platelets (Table III)

No obvious correlation was found between inhibition of uptake of ^{3}H NA in the platelets by α receptor blocking agents and their effect on amine induced platelet aggregation It therefore appears as if uptake of amines is not a necessary step in catecholamine mediated platelet aggregation and that uptake and aggregation were mediated through different mechanisms The inhibition of uptake was observed with some α and β receptor blocking agents in concentrations from 10^{-6} M At higher concentration levels the uptake of 5 HT was also inhibited indicating a more unspecific effect in these cases

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Intracellular Distribution of Lipase in Comparison to Trypsinogen, Amylase and Immediately Measureable Trypsin Inhibitor(s) in the Rat Pancreas

By

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Abstract

Arnesjo, B and A Grubb *Intracellular distribution of lipase in comparison to trypsinogen, amylase and immediately measureable trypsin inhibitor(s) in the rat pancreas* Acta physiol scand 1969 75 139—148

The intracellular distribution of lipase (glycerol ester hydrolase EC 3 1 1 3), trypsinogen (EC 3 4 4 4), amylase (EC 3 2 1 1) and immediately measurable trypsin inhibitor(s) has been studied using isopycnic gradient centrifugation of rat pancreatic homogenates A modified method for the measurement of trypsinogen is presented and a micromethod for the estimation of trypsin inhibitory activity is described By comparison with lipase smaller proportions of the total trypsinogen and amylase activities were recovered from the zymogen fractions The total immediately measurable trypsin inhibitory activity was mainly localised in the cytomembrane fractions while virtually no activity was found in the zymogen granules The specific activity (activity per mg protein) for both amylase and trypsinogen was high in the zymogen and in the cell sap fractions For lipase and immediately measurable trypsin inhibitory activity however the specific activity was high only in the zymogen granules and cytomembranes respectively

In a recent report we showed that at least 25—45 % of the lipase cholesterol esterase phospholipase activities and that an esterase activity that can be stimulated by bile salt are concentrated to the zymogen fractions obtained after isopycnic gradient centrifugation of rat pancreatic homogenates (Arnesjo and Filipek-Wender 1968)

Regarding the intracellular distribution of the proteolytic enzymes present in the pancreatic tissue in the forms of inactive precursors the previous results have differed considerably because activation of the precursors has been complicated by the presence of protease inhibitors Hokin (1955) found that spontaneous activation of all protease zymogens can occur in zymogen granules isolated from dog pancreatic homogenates On the other hand Siekewitz and Palade (1958) found almost no spontaneous activation of the total protease precursors in homogenates or subfractions obtained from the guinea pig pancreas The same authors claimed that activation of the homogenate or the obtained subfractions could be nearly total by the addition of

a catalytic amount of trypsin In spite of this they could provide evidence for the presence of trypsin or protease inhibitory activities in the final supernatant and in the homogenate when the same amount of activator was used Therefore they presumably did not measure the content of total protease zymogens present in the fractions with inhibitory activities The authors found about 40 % of the recovered total trypsin activable protease activity in the zymogen granule fractions In a study of the subcellular distribution of trypsinogen in the guinea pig pancreas Hansson (1959) has essentially confirmed the findings by Siekewitz and Palade

In reports of the amylase activity distribution between the subcellular components of the pancreas the results vary considerably In general, however, the amount recovered from the zymogen fraction is smaller than in the case of proteases—most of the enzyme activity being associated with the microsomes or the final supernatant (Hokin 1955 Hansson 1959, Schramm 1967, Laird and Barton 1957, Laird and Barton 1958 van Lanker and Holtzer 1959)

The presence of protease inhibitory activities in pancreas and pancreatic juice is well known (Vogel *et al* 1966) It is also well established that the trypsin inhibitory activity in the pancreatic juice is correlated with its protein content and not with its total volume (Kalser and Grossman 1955 Green *et al* 1966, Fritz *et al* 1967) Zymogen granules contain part of the proteins (including protease precursors) secreted in the pancreatic juice These two facts have lead several authors to suggest that also the trypsin inhibitory activity is mainly localised in these granules (Green *et al* 1966 Fritz *et al* 1967)

This investigation was undertaken in order to study by isopycnic gradient centrifugation of rat pancreatic homogenates the intracellular distribution of lipase by comparison with amylase and trypsinogen In the latter case the activities should be measured with consideration of the presence of trypsin inhibitors for the estimation of which a reliable method had to be found This method could then also be used to study directly the subcellular distribution of trypsin inhibitor(s)

Materials and methods

Chemicals

All chemicals used were of reagent grade purity

Trypure Novo (crystalline trypsin) was purchased from Novo Industri A S Copenhagen

Benzoyl dl arginine p-nitroanilide HCl (DL BAPA) was purchased from Nutritional Biochemical Corporation Cleveland Ohio

Toluene p-sulphonyl l arginine methyl ester HCl (TAME HCl) was purchased from BDH Poole England

Bovine serum albumin was purchased from AG Serva Heidelberg

Triton X 100 was purchased from Sigma Chemical Company St Louis Missouri

The final pH adjustments of all buffers used were made with a pH meter (Radiometer Copenhagen)

Methods

Protein was measured by the method of Lowry *et al* as modified by Eggstein and Kreutz (1955) and phospholipid phosphorus by a modified version (Belfrage 1967) of the method for the determination of inorganic phosphate according to Itaya and Ui (1966)

Enzyme assays

Lipase activity was assayed as previously described (Arnesjo and Filipek Wender 1968)

Amylase activity was estimated as described by Dahlqvist (1962)

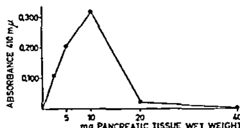


Fig 1 Activity versus concentration of activated ... wet ... dis ... pH 7 6 0 02 M with respect to $CaCl_2$ (For further details see methods)

Trypsin actuable trypsin activity (trypsinogen) was measured in the following assay system 0 2 ml fraction to be tested was activated by incubation for 48 hours at 0—4° C with 0 8 ... t to $CaCl_2$ ... of the cold ... and Cohen ... trypsin was determined in the same system except that the preincubation was carried out in the buffer alone The small amount of trypsin used to activate the trypsinogen gave by itself negligible hydrolysis of the substrate under the assay conditions Centrifugation of the activated mixtures before or after incubation with the substrate gave the same measurements ...

activated by 1 6 μg trypsin (Fig 1) The aliquots for testing tryp... pancreatic homogenates thus must be chosen very carefully in order to record true values for the amount of trypsinogen present

Immediately measurable trypsin inhibitory activity The trypsin inhibitory activity of the pancreatic homogenates and subfractions was estimated by measuring its ability to lower the hydrolysis rate produced by a standard amount of trypsin in the assay system described by Hummel (1959)

All reagents were freshly prepared The fractions to be tested were if necessary diluted with 0 88 M sucrose to give a suitable degree of inhibition—*i e* below 70 % (Vide infra) All ... performed at 30° C in a thermostatically controlled cuvette holder installed ... The substrate solution and the two cuvettes ... 30° C before use while the trypsin standard ... *tris HCl buffer pH 8 1 being 0 02 M with* respect to $CaCl_2$) and the fractions to be tested were kept at 0 C

The measuring procedure was as follows

To the test cuvette containing 1 2 ml substrate solution was added 0 1 ml of the fraction to be tested and immediately thereafter 0 1 ml of the trypsin standard solution (=0 225 μg trypsin)

To the reference cuvette containing 1 2 ml substrate solution was also added 0 1 ml of the fraction to be tested and immediately afterwards 0 1 ml cold buffer instead of the trypsin standard solution The consecutive additions to the two cuvettes were performed almost simultaneously

After shaking for about 45 sec the increase in absorbance at 247 mμ wavelength (Hummel 1959) was continuously registered on a damped recorder (Aldo and Arvidsson 1968) The curves were rectilinear for 15 min The degree of inhibition was expressed as μg trypsin inhibited and calculated as described by Trautschold (*Vogel Trautschold and Werle 1966*) in reference to a trypsin standard curve where 0 1 ml cold 0 88 M sucrose was substituted for the fractions to be tested

The trypsin inhibitory activity is directly proportional to the added wet weight of homogenized pancreatic tissue up to at least 70 % inhibition (SD = 0 008 μg trypsin inhibited) The substitution of 1 mg albumin dissolved in 0 1 ml cold 0 88 M sucrose for the fractions to be tested caused no non specific inhibition The protein content of the homogenates or the subcellular fraction aliquots tested in this study was below 1 mg

Preparation of homogenates and fractionation was performed as described before (Arnesjö and Filipek Wender 1968 with the exception that 0 88 M sucrose was substituted for 0 25 M sucrose as the homogenizing medium (Hogeb...

TABLE I Release of trypsinogen, lipase, amylase and trypsin inhibitory activity in rat pancreatic homogenates (For details see Methods)

Treatment	Trypsinogen		Lipase		Amylase		Trypsin Inhibitor(s)	
	Zymogen Fraction	Supernatant	Zymogen Fraction	Supernatant	Zymogen Fraction	Supernatant	Zymogen Fraction	Supernatant
Triton X 100	100 0	100 0	100 0	100 0	100 0	100 0	100 0	100 0
Freezing and thawing twize	84 7	98 6	100 3	98 8	100 6	99 8	116 4	99 0
No treatment	89 5	104 6	103 0	98 8	98 2	100 3	103 7	99 1

In separate experiments the supernatant obtained after centrifugation of the homogenate for 10 min at $700 \times g_{av}$ was further centrifuged for 20 min at $4300 \times g_{av}$ (Siekevitz and Palade 1958) The sediment was diluted with the same volume of 0 88 M sucrose as the supernatant The two fractions thus obtained were tested for lipase, trypsinogen, amylase and immediately measurable trypsin inhibitory activity with and without previous treatment with 10 % (v/v) 2 % (v/v) triton X 100 and with freezing and thawing twice Neither of the treatments did increase the measured enzyme or inhibitory activities (Table I)

Results

Morphological examinations

Macroscopically the distribution of the different opalescent layers in the gradient tube after centrifugation ($135{,}000 \times g_{av}$ 2 hrs) of the rat pancreatic homogenates was the same as described before Also at examination in the light microscope using phase contrast optics the distribution appeared to be the same and thus the fractions within density ranges 1 240—1 210, 1 210—1 183 and 1 183—1 140 shall be referred to as zymogen granule mitochondrial and membrane-cell sap fractions respectively (Arnesjo and Filipek-Wender 1968)

Chemistry

0 88 M sucrose was chosen instead of 0 25 M sucrose as the homogenizing medium as we had observed when using this higher concentration that smaller losses of zymogen granules and enzyme activities occurred to the sediment obtained after centrifugation of the pancreatic homogenates $700 \times g_{av}$ for 10 min This medium however gave higher measurements of protein and enzyme activities in the cell sap fractions as compared with the other subcellular fractions indicating a poorer preservation of the different particle populations in this medium Compared to previous results a maximal shift to the cell sap of 10 % for lipase activities and 5 % for protein was observed

TABLE II Percent distribution and recovery of protein, enzymes and trypsin inhibitory activity after equilibrium density centrifugation of rat pancreatic homogenates

Homogenates from overnight fasted rats containing 1 g tissue wet weight/5 ml 0 88 M sucrose were centrifuged at 700 × g_{av} for 10 min 0 4 ml of the resulting supernatant was applied on top of a tube containing the linear aqueous sucrose gradient and then centrifuged in a swing out head for 2 hrs at 135 000 × g_{av}

Measured	Applied	Recovered	Expressed as	Recovery %	Per cent recovered at densities		
					1 240—1 211	1 203—1 183	<1 174
Protein	14 158±0 932	11 374±0 581	mg	60 5±5 0	9 3±2 9	10 1±2 1	80 7±4 5
Lipase	243 4±16 9	174 4±9 9	μmoles fatty acid released per 2 min	71 7±1 7	31 7±2 2	14 6±1 2	55 2±1 9
Trypsinogen	759 4	669 2±109 0	μg trypsin	88 5	15 2±5 4	5 5±2 8	79 2±6 6
Amylase	1 103 4±273 0	944 5±220 0	mg maltose released per 3 min	85 9±3 1	12 2±1 9	6 9±1 4	82 5±2 9
Trypsin inhibitor(s)	7 255	5 836	μg trypsin inhibited	80 6	4 8	7 1	88 4

Protein

The mean protein content in the rat pancreatic glands was found to be 177±12 mg per gram tissue wet weight The protein distribution presented in Table II and Fig 2 is in agreement with previous results except for a small shift to the cell sap fractions

Phospholipids

In one experiment the distribution of phospholipids in the test gradients was measured This is shown in Fig 2 Obviously the zymogen granule fractions and the cell sap contain most of the nonstructural protein since phospholipids are mainly localized to membranes The results also indicate that the main part of the cytomembranes is concentrated at an equilibrium density of 1 156 which is in agreement with previous election microscopical results

Fn-yme actu ities

Lipase Table II and Fig 3 show the distribution of lipase activity This forms the same pattern as described before with activity maxima confined to the zymogen granule and membrane-cell sap fractions and with the highest specific activity (i e activity per mg protein) in the zymogen granules

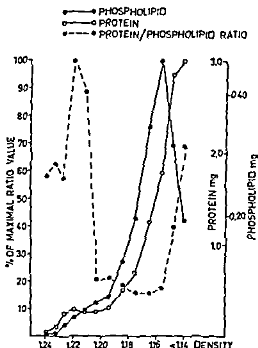


Fig 2 Distribution of protein phospholipid and protein phospholipid ratio after isopycnic gradient centrifugation of rat pancreatic homogenates

Trypsinogen No spontaneous trypsinogen activation has been observed even after prolonged storage of the homogenates or the subcellular fractions in the cold or at room temperature for periods of up to 4 days

The pattern of distribution for trypsinogen versus fraction density is presented in Table II and Fig 3 Two main localizations can be observed—one in the zymogen granule—and one in the membrane cell sap fractions In comparison with lipase however the main part of the trypsinogen in the latter fractions is shifted more towards the lowest densities and thus trypsinogen is more concentrated in the cell sap than to the membranes where the largest fraction of the lipase activity was recovered

15 2 ± 5 4 % of the recovered trypsinogen activity was found in the zymogen granule fractions which figure is significantly smaller than that of lipase However the total trypsinogen recoveries from the pancreatic subfractions were higher than those for lipase which might cause errors when comparing the distributions of the two enzymes These errors however assumed to be maximal did not account for the differences between the percentage distributions

The specific activities for trypsinogen in the different subcellular structures form a pattern different from lipase with high figures in the zymogen granules and in the cell sap A small but significant peak was found in the membrane fractions

Amylase The recovered amylase activity revealed a pattern of distribution strikingly similar to that of trypsinogen The small differences in the percent distributions were not significant Fig 3 and Table II show the distribution of amylase activity to the pancreatic subcellular components

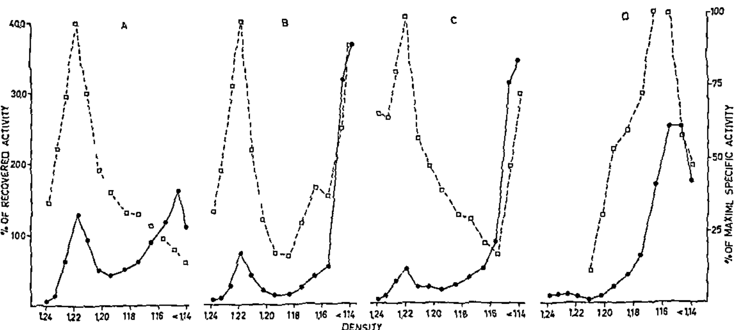


Fig. 3. Distribution of total and specific activities for lipase (A), trypsinogen (B), amylase (C) and immediately measureable trypsin inhibitor(s) (D) after isopycnic gradient centrifugation of rat Pancreatic homogenates.
●——● total activity □——□ specific activity (The data represent the mean in at least three experiments.)

Immediately measurable trypsin inhibitory activity (IMTIA) The straight line relationships between increase in absorbance and time during the first 15 min indicates a negligible activation of trypsinogen in the tested fractions during this time by the small standardized amounts of added trypsin

The mean content of IMTIA in the homogenized pancreas from over night fasted rats is 90 7 µg trypsin inhibited per gram tissue wet weight This activity is distributed to the subcellular structures according to Table II and Fig 3 The highest activities were present in the membrane fractions where a peak of activity was found In the zymogen fractions virtually no IMTIA could be estimated

The specific IMTIA (activity per mg protein) formed a peak confined to the cytomembranes where the contamination by the cellsap is small or negligible (Fig 2 and 3)

In this investigation the IMTIA has been exceeded by at least one µg trypsin when activating the trypsinogen present in the pancreatic homogenates and the sub fractions The trypsinogen in an aliquot containing 5 mg of pancreatic tissue wet weight can be maximally activated by 1 6 µg trypsin (Vide supra) Since the same aliquots have been used and since the concentrations of both IMTIA and trypsinogen is smaller than in the homogenate it can be concluded that all the subfractions have been maximally activated when testing for the presence of trypsinogen therein This *conclusion is further supported by the total recoveries of approximately 90 % from* the testgradient fractions

Discussion

As previously shown isopycnic gradient centrifugation is a useful method for studies of intracellular distributions of digestive enzymes in the rat pancreas (Arnesjo and Filipek Wender 1968 The particle populations cannot be completely separated by this method but the intercontaminations are less extensive than in the subcellular fractions obtained by differential centrifugation procedures In particular the contamination by the cell sap is negligible The cell sap contains high amounts of protein and enzymic activities which can be derived from different sources e g pancreatic juice proteins present in the ducts proteins released from particles during homogenization and fractionation blood derived proteins and proteins originally dissolved in the cytoplasm The importance of these four possible sources cannot be further elucidated which means that the absence of cell sap contamination in the particle fractions is an advantage in this study

In this investigation the pancreatic lipase activity had almost the same pattern of distribution subcellularly as previously presented Arnesjo and Filipek Wender 1968

The distribution of trypsinogen is different from that of lipase Only 15 % of the pancreatic trypsinogen was recovered in the zymogen granule fractions where 30 % of the lipase activity was found In view of the results obtained in this and previous investigations it can probably be concluded that in relation to trypsinogen larger proportions of the lipase cholesterol esterase and phospholipase in the pancreatic

exocrine cells are localized in the zymogen granules These conclusions are contradictory to the findings of Hokin Hansson and Siekewitz and Palade, which contradiction, however, presumably can be explained by the fact that these authors did not measure protease precursors in the fractions where activator inhibitors were present (Hokin 1955, Palade *et al* 1962, Hansson 1959) Another explanation might be that after release during homogenization higher amounts of all three lipolytic enzymes than of trypsinogen are readsorbed at the surface of the zymogen granules Such readsorption of chymotrypsinogen at microsomes has been reported by Keller and Cohen (1961) An equivalent explanation, however, seems less applicable here since rat pancreatic juice trypsinogen and cholesterol esterase are anionic proteins while lipase, amylase, phospholipase and trypsin inhibitor(s) are cationic (Barrowman *et al* 1968) Electrostatic adsorption thus probably cannot account for the differences in the enzymic distributions

The amylase activity distribution does not differ from that of trypsinogen About 12 % of the total amylase activity was found in the zymogen granules This figure corresponds well with previously published results (Hokin 1955, Hansson 1959, Laird and Barton 1957, Laird and Barton 1958, Lanker and Holtzer 1959)

The detailed distribution of the enzymes studied between the membranes, (rough and smooth surfaced membranes) free ribosomes and cell sap cannot be firmly established The results indicate, however, that lipase is more concentrated to the particles of these fractions than either amylase or trypsinogen.

We consider it convenient to subdivide the pancreatic cellular pool of trypsin inhibitory activity as follows An accessible pool and an inaccessible pool part of which has reacted with trypsin thus forming inactive trypsin—trypsininhibitor complexes, which can be quantitated (Kalser and Grossman 1955, Fritz *et al* 1966) The remaining part of the inaccessible pool cannot at present be estimated The inaccessible pool is not determined by the method used in this study The treatments of the pancreatic homogenates or subfractions in order to further release inhibitory activities did however not change the total content of accessible trypsin inhibitory activity Neither did the activity diminish after prolonged storage of the inhibitor sources during which a spontaneous release of trypsin could be expected These two facts indicate that the part of the inaccessible pool which has not reacted with trypsin is small under the ordinary assay conditions and also that at least at 0—4° C the rate of spontaneous trypsinogen activation is extremely low

Almost all of the immediately measurable trypsin inhibitory activity was found in the membrane cell-sap fractions with a peak in those fractions where almost only membranes and the highest concentrations of phospholipids were observed Furthermore the specific activity distribution indicates that the inhibitory activity towards trypsin is mainly associated with rough surfaced membranes In the zymogen granule fractions only very small or no activities could be found

It is curious that the distributions of an autocatalysing proenzyme and its inhibitor so do not correspond These distributions however, might have a physiological explanation if trypsinogen present in zymogen granules is subjected to less risk of

activation than the trypsinogen under transport through the cell from its site of formation. This difference, in turn might be due to differences in the spatial organisation between enzyme inside and outside the zymogen granules

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The Effects of Extracellularly Applied ATP and Related Compounds on Electrical and Mechanical Activity of the Smooth Muscle Taenia Coli from the Guinea-Pig

By

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Abstract

AXELSSON, J and B HOLMBERG *The effects of extracellularly applied ATP and related compounds on electrical and mechanical activity of the smooth muscle taenia coli from the guinea pig* Acta physiol scand 1969 75 149—156

The effect of external ATP and related compounds on the electrical and mechanical activity of isolated [illegible] of taen[illegible] has been [illegible] ATP AMP [illegible] the sp[illegible] ATP [illegible] GMP [illegible] compounds and adenine which was the most potent Here again GMP guanosine and inosine had no effect ATP AMP and adenosine prevented or shortened the initial depolarization produced by acetylcholine and carbachol Inorganic phosphate was liberated from ATP and AMP applied to the bathing solution The hydrolysis was not significantly decreased by g strophanthin p-Hydroxy mercuribenzoate caused an initial excitation as well as g strophanthin but neither inhibited the relaxing effect of ATP The effects of extracellularly applied ATP are consistent with the view that they are exerted through changes in membrane permeabilities and that some of these effects are brought about by the adenosine part of the molecule

Drury and Szent Gyorgy (1929) discovered that adenosine and related compounds affected the mechanical activity of various tissues A comprehensive account of the complex pharmacological effects of these compounds which include relaxant actions was given by Gillespie (1934)

Extracellularly applied ATP has been found to inhibit spontaneous spike discharge and also the contractures maintained by the depolarized taenia coli (Axelsson *et al* 1965 Bueding *et al* 1967) The present experiments were undertaken to try to determine whether these actions are due to ATP supplying chemical energy to the cell membrane or to other actions exerted by adenosine and related compounds on the cell membrane

We have studied 1 The effects of ATP AMP adenosine adenine, GMP, guanosine and inosine on spontaneous electrical and mechanical activity and on con —

tractures 2 Some effects of ATP-ase inhibitors 3 The liberation of inorganic phosphate from ATP and AMP applied to the bathing solution

Some of the results have been presented to a meeting of the XII Scandinavian Congress of Physiology (Axelsson *et al* 1966)

Methods

Dissection and pretreatment

Guinea pigs of both sexes were stunned with a blow and bled out The caecum was immersed in normal Krebs solution at 37° C with adrenaline 2×10^{-8} g/ml to abolish spontaneous activity Pieces of taenia coli of about 25 mm were dissected from the caecum freed of connective tissue and transferred to a dish containing the dissecting solution Small stainless steel hooks were tied to each end of the muscle and used to attach one end of the tissue to a plastic rod and the other end to a force displacement transducer via a plastic pin The length of the muscle could be changed with a micrometer screw Each muscle mounted in this recording apparatus was transferred to a bath containing 50 ml normal Krebs solution and adrenaline 2×10^{-8} g/ml at 37° C The inactive in vitro length (L_0) was determined (Aberg and Axelsson 1965) and the muscle was allowed to recover for one hour in normal Krebs solution (Goodford and Hermansen 1961 The inactive in vitro length was again determined in the presence of adrenaline before beginning the experiment All experiments were done at 37° C

Solutions

The compositions of the various solution are described in the table

Solution (mM	K^+	Na^+	Ca^{2+}	Mg^{2+}	Cl	HCO_3	H_2PO_4	Glucose
Normal	5 93	137 47	2 49	1 19	134 11	15 48	1 19	11 5
High K^+	127 94	15 48	2 49	1 19	134 11	15 48	1 19	11 5
Phosphate free	5 93	137 47	2 49	1 19	135 30	15 48	0	11 5

The solutions were prepared with twice distilled water and chemicals of Pro Analysi quality They were bubbled with a mixture of O_2 (97 %) and CO_2 (3 %) and pH was about 7 4

Chemicals and drugs

ATP Adenosine 5 triphosphate disodium salt AMP Adenosine 5 monphosphoric acid and sodium salt GMP Guanosine 5 monophosphoric acid and sodium salt Adenosine Adenin Guanosine Inosine p-Hydroxymercuribenzoate sodium salt (Sigma) g Strophantin (Sandoz Carbacholinchloride Merck Acethylcholinchloride Siegfried)

Any changes in pH occurring when the nucleotides were dissolved were adjusted by bubbling the solutions with the gas mixture for 15 min before beginning the experiment

Recording technique

The force displacement transducer used for isometric recording of muscle tension was Grass FT 03 The output potential was recorded on Beckman Offner Dynograph R B

For simultaneous recording of electrical and isometric mechanical activity we used the sucrose-gap method Stampfli 1954 Burnstock and Straub 1958

Determination of inorganic phosphate

Eight or nine pieces of taenia from the same animal were used After determination of L_0 the muscles were transferred to phosphate free Krebs solution for 1 hr Thereafter each muscle was incubated in a bath containing 10 ml phosphate free Krebs solution and $1\ 65 \times 10^{-3}$ M ATP After 1 hr inorganic phosphate in the solution was determined with a method described by Berenblum and Chain and modified by Martin and Doty (1956 The phosphomolybdic acid was extracted with isobutyl alcohol and separated from the easily hydrolysable phosphate compound (ATP)

The hydrolysis of ATP in the solution under the same conditions but without muscle was always measured in the same experiment After exposure to phosphate free Krebs solution for one hour the phosphate coming from the muscle into the solution was negligible

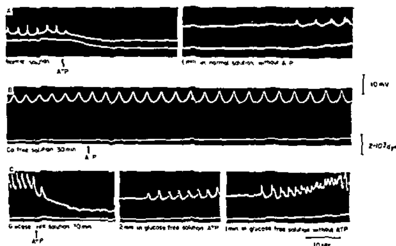


Fig 1 The effect of ATP (1.5×10^{-3} M) on electrical and mechanical activity in various solutions A Normal Krebs solution B After 30 min in Ca free solution C After 30 min in glucose free solution Upper record Electrical activity Lower record Tension

Results

Ia The effect of ATP on spontaneous electrical and mechanical activity

Mechanical activity was recorded isometrically as described in Methods After 1 hr recovery in normal solution the muscles showed varying degrees of spontaneous activity ATP was then applied to the bathing solution At L_0 ATP in concentrations from 1 to 5 × 10 [illegible] M caused inhibition of tone and when ATP was washed out the muscle usually became hyperactive The duration and degree of inhibition was quantitatively related to the concentration of ATP and inversely to the initial stress With concentrations ranging from 10 [illegible] M to 1.5 × 10 [illegible] M the inhibition was fully reversible Simultaneous recording of electrical and mechanical activity showed that the relaxation was preceded by inhibition of spike discharge and hyperpolarization Fig 1a (See also Axelsson *et al* 1965 1966 Bueding *et al* 1967

In Ca free solution mechanical activity *of the tissue is abolished before the* electrical activity ceases (Axelsson and Bülbring 1959 Axelsson 1961 At this stage the electrical activity consists of slow potential changes and addition of 1.5×10^{-3} M ATP *caused no inhibition of this discharge (Fig 1b)* This has been previously reported by Axelsson *et al* (1966) and Bueding *et al* (1967)

Removing glucose from the bathing solution results in an increased frequency of spike discharge while the mechanical response of the tissue is gradually abolished This has been quantitatively described by Axelsson *et al* (1965) After 30 min exposure to glucose free solution ATP (1.5 mM) still inhibited the spike discharge and caused hyperpolarization which however was shortlasting and the spontaneous spike discharge reappeared in the presence of ATP (Fig 1c)

Ib The effects of ATP on contractures

Contractures are here defined as prolonged reversible c

opments which are not maintained by action potentials After one hour in normal solution, contractures were initiated by depolarizing the tissue, either by replacing the extracellular sodium with potassium or by adding acetylcholine or carbachol to the bathing solution

High K contractures

The concentration of potassium which was used depolarized the membrane beyond the level for initiation and conduction of spikes The effect of ATP on the contractures was studied ATP was added after 1 hr in high K^+ solution when the contractures were approximately stable and were 58 ± 9 % of the maximum initial tension developed in response to depolarization ATP always relaxed the contractures, its effect was fully reversible and graded according to dose

After 5 min exposure to high K^+ solution containing 1.5×10^{-3} M ATP, the contractures were reduced by 38 ± 9 % (see Table II) The effects of lower concentrations of ATP are described in section 4

Acetylcholine and carbachol contractures

Burnstock (1958) noted a repolarization of the membrane during continued exposure to high concentration of ACh and this finding has been confirmed and extended ACh and carbachol in concentrations up to 1×10^{-4} g/ml caused depolarization with increased frequency of spike discharge followed by cessation of spikes In spite of the continued presence of the drugs this was followed by a slow repolarization and return of spike activity with fluctuations in mechanical tone (Axelsson and Wahlström 1966) Contractures as defined above were therefore only maintained for a short period (ca 2 min in presence of 10^{-4} g/ml carbachol)

The effect of ATP was studied in three different ways either ATP was administered one minute before the depolarizing drug or one minute after administration when depolarization was maximal and contracture maintained or ATP was applied simultaneously with the depolarizing drug In the first type of experiment ATP prevented or shortened the initial depolarization and the duration of the mechanical response In the second type when ATP was added at the height of depolarization

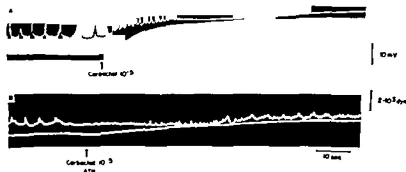


Fig 2 Simultaneous recording of electrical and mechanical activity of the effect of A Carbachol 10^{-5} g/ml B Simultaneously administration of carbachol 10^{-5} g/ml and ATP 1.5×10^{-3} M

TABLE I Hydrolysis of external ATP and AMP by taenia coli during 1 hr incubation. Muscle length 19—22 mm

Muscle wet weight	Liberated inorg. phosphate (μmole)		
	ATP	ATP + 10^{-4} g/ml g strophanthin	AMP
8.0—11.9 mg	2.2 ± 0.5 (10)	2.5 ± 0.2 (4)	—
12.0—15.9 mg	2.4 ± 0.5 (9)	2.6 ± 0.4 (6)	1.0 ± 0.2 (6)

Means ± S.D. Number of experiments in brackets)

a repolarization occurred and slow waves were initiated. These waves became gradually more rapid and finally spike discharge associated with fluctuation in tone appeared. Simultaneous administration of ATP and the depolarizing drug reduced the effect of carbachol to a small increase in spike frequency. The effect of simultaneous administration is shown in Fig. 2b.

2. Some effects of ATP-ase inhibitors

The Na^+ and K^+ dependent ATP-ase activity of human red cells is inhibited by g-strophanthin (Dunham and Glynn 1961). The Na^+ and K^+ activated ATP hydrolysing enzyme system from ox brain is inhibited by g-strophanthin and p-hydroxymercuri benzoate (Skou 1963).

In the present experiments p-hydroxymercuri benzoate (10^{-}[illegible] g/ml) caused an increase in the frequency of spontaneous spike discharge and had a relaxing effect on the high K contractures but did not abolish the relaxing effects of ATP.

The initial effect of g-strophanthin (10^{-4} g/ml) was an increase in spontaneous spike discharge consistent with the findings of Schatzmann and Ackermann (1961) and Casteels (1966). After about 20 min the spike discharge stopped but addition of ATP still produced an increase in the membrane potential. g-Strophanthin had no effect on the high K contractures and did not inhibit the relaxing effect of ATP.

3. Liberation of inorganic phosphate from externally applied ATP and AMP

ATP in the incubation medium was 1.65×10^{-3} M and the muscles were incubated for one hour. The results are shown in Table I.

Because of different geometry of the muscle pieces and consequent differences in diffusion the muscles were divided into groups with respect to weight. See Table I.

In each experiment the hydrolysis of ATP in the solution was measured under the same conditions but without muscle. In the table all figures for liberated phosphate are corrected for this amount (ca 25 %). As pointed out in Methods the phosphate coming from the muscle into the solution was negligible. Thus the presence of a muscle gives rise to considerable breakdown of ATP in addition to the normal hydrolysis at 37° C. AMP in contrast to GMP in concentration 1.65×10^{-3} M was hydrolysed under the same conditions (Table I). The splitting of ATP was not inhibited

by g strophanthin 10^{-4} g/ml. (This concentration was found by Dunham and Glynn (1961) to inhibit the ATPase activity of human red cells.) Nor did p-hydroxymercuri benzoate inhibit the splitting of ATP by the tissue. The hydrolysis in phosphate free solution in absence of tissue was not affected by previous incubation of a muscle in that solution for 1 hr.

(c) The effects of AMP, adenosine, adenine, GMP, guanosine and inosine compared with the effect of ATP on spontaneous electrical and mechanical activity and on contractures

None of the nucleotides altered the pH of the Krebs solution by more than 0.1 unit. Adenosine and adenine in the same concentration dissolved in Krebs solution had no effect on pH. To determine the effects of reduced pH both Na-salt and acid of AMP were tested in the experiments and no difference in relaxing effect was seen. AMP and adenosine inhibited spontaneous electrical and mechanical activity in a manner similar to ATP (see section 1a). Adenine had no or a transient effect and GMP, guanosine and inosine were without effect on a spontaneously active muscle. When ATP, AMP and adenosine (1.5 mM) were applied to a muscle spontaneously maintaining high frequency of spike discharge ATP produced the most long lasting inhibition. The lowest concentration of the various compounds causing relaxation of a spontaneous active muscle were: ATP 1 to $5 \times 10^{-[illegible]}$ M, AMP 0.5 to $1 \times 10^{-[illegible]}$ M, adenosine 0.5 to $1 \times 10^{-[illegible]}$ M.

Carbachol ($10^{-[illegible]}$ g/ml) produced no contracture but the muscle was hyperactive and the effect of the various compounds were studied after one minute. ATP transiently inhibited or reduced the frequency of spike discharges and consequently the tension fell. AMP and adenosine reduced the frequency of spike discharges and caused a small decrease in tension (Fig. 3). Adenine had no effect. The lowest relaxing concentrations were: ATP 0.5 to 1×10^{-6} M, AMP 0.1 to 0.5×10^{-3} M, adenosine 0.1 to 0.5×10^{-3} M.

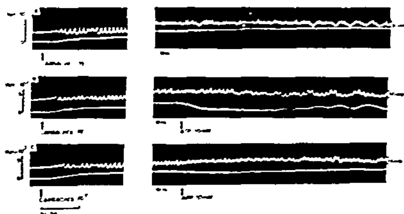


Fig. 3. The effect on electrical and mechanical activity of: A, Carbachol 10^{-7} g/ml; B, Carbachol 10^{-7} g/ml and ATP 1.0×10^{-3} M after 1 min; C, Carbachol 10^{-7} g/ml and AMP 1.0×10^{-3} M after 1 min.

TABLE II % reduction of high K^+ contracture induced by 1.5×10^{-3} M adenine compounds

ATP	AMP (Na salt)	Adenosine	Adenine
33 ± 9 %	36 ± 12 %	43 ± 13 %	71 ± 7 %

Means ± S.D. Number of experiments 14

In a series of 14 expts the relaxing effects of ATP, AMP, adenosine and adenine (in conc 1.5 mM) on high K contractures were studied by adding the compounds after 1 hr in high K solution when the contractures were approximately stable. The relaxing effect is expressed as the percentage reduction of tension 5 min after application of the drug. The relaxation was fully reversible and the muscles were washed in high K solution during 10 min between the test solutions. The contracture decreased only about 5—10 % between the test periods and the relaxing effect of the various compounds was independent of the sequence of adding. The results are given in Table II.

In all the muscle studied adenine was the most potent relaxant of the potassium contracture. The order of potency was adenine > adenosine > AMP ≅ ATP.

The lowest relaxing concentrations were ATP 0.1 to 0.5×10^{-3} M, AMP 0.1 to 0.5×10^{-3} M, adenosine 0.1 to 0.5×10^{-4} M and adenine 0.1 to 0.5×10^{-3} M.

Guanosine and inosine were found to have no relaxing effect on high K contractures.

Discussion

These results indicate that the action of ATP in relaxing potassium contractures may be produced by the adenine part of the molecule and not by the ability of ATP to supply chemical energy through its terminal phosphate group. Potassium contractures were relaxed at least as readily by equimolar concentrations of other adenine compounds as by ATP. Our finding that adenosine does relax potassium contractures conflicts with a report by Imai and Takeda (1967) but the negative finding by those authors can be explained by the lower concentration of adenosine that they used. Since potassium contractures are believed to be due to inward diffusion of calcium into the cells (Durbin and Jenkinson 1961), the action of adenine compounds on such contractures might be brought about by their interference with this process.

The lack of correlation between the number of terminal phosphate groups and the relaxing effect of adenine compounds on potassium contractures was not equally obvious in other experimental situations. In normal solution, for example, ATP inhibited spike frequency, hyperpolarized the membrane and caused relaxation in very much lower molar concentrations than other adenine compounds.

The fact that both g-strophanthin and p-hydroxymercuri benzoate failed to inhibit this action of ATP make it unlikely, however, that it was exerted through an increased supply of energy. But then it must also be remembered that the hydrolysis of ATP was uninfluenced by these agents. Although it may be argued from these ..

ments that ATP and related compounds may exert their effects extracellularly interfering with normal membrane processes, we have found that muscles incubated with ^{14}C-ATP incorporated the labelled part of the molecule (unpublished), therefore an extracellular effect cannot be excluded

We are grateful to Miss Gunilla Eriksson for excellent technical assistance and G Hogberg for some of the sucrose gap records

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Increased Vascular Permeability Caused by the Trypsin-like Enzymes Purified from Rat Submandibular Gland

By

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Abstract

Ekfors, T O, V K Hopsu-Havu and T Malmiharju *Increased vascular permeability caused by the trypsin like enzymes purified from rat submandibular gland* Acta physiol scand 1969 75 157—160

the enzymes effect the vascular permeability by liberating vasoactive kinins

The purification and characterization of three enzymes resembling pancreatic trypsin from the rat submandibular gland have been reported earlier (Riekkinen, Ekfors and Hopsu 1966, Riekkinen, Ekfors, Hollmen and Hopsu-Havu 1967, Riekkinen, Ekfors and Hopsu-Havu 1967, Ekfors, Riekkinen, Malmiharju and Hopsu-Havu 1967) The first one of the enzymes was *salivain*, a proteinase hydrolysing readily the synthetic as well as natural protein substrates of pancreatic trypsin Its pH optimum is at 9 3 and it is not inhibited by ovomucoid and lima bean trypsin inhibitors *Glandulain*, the second enzyme, hydrolyses the same substrates at comparable rates having pH optimum at 8 1, and it is inhibited by the usual trypsin inhibitors Alltogether four isozymic forms were identified in the third enzyme called *kallikrein like peptidase* This enzyme hydrolyses the esters and amides of N-substituted arginine optimally at pH 8 2, while proteins are hydrolysed only minimally This enzyme was found to be inhibited by trypsin-inhibitors only to a slight degree Both salivain and the kallikrein-like peptidase, but not glandulain, were shown to be highly hypotensive in rabbits (Ekfors, Malmiharju, Riekkinen and Hopsu-Havu 1967) This effect may be due to a kinin-liberating effect of the enzymes with serum

α_2-globulins as substrates The results of this paper show that the same enzymes are also capable of increasing the capillary permeability when injected intradermally in blued rabbits

Experiments

The back of adult rabbits weighing 2—3 kg was depilated by using an animal clipper The area extended 3—4 cm on both sides of the dorsal midline between hips and shoulders Filtered 0 5 % Evans Blue in saline was injected in the ear vein Intradermal injections of the various test substrates were carried out in 0 05 ml volumes with fine needles 5 min later The lesion indicated by the blue colour extravasated around the site of injection was recorded 30 min later All of the enzymes and [illegible] blueing due to mechanical injury caused by the injection.

In the test of the effect of antihistamine drugs, prometazine (1 mg kg Phenergan ®, M&B) was injected i v The method described by Bhoola *et al* (1960) was followed

To test the effect of phenylbutazone on the reactions Butazolidin ® (J R Geigy A G, 125 mg kg) was injected intramuscularly 12 and 2 h before the experiment

Results

A typical test result is represented in Fig 1

Salivain was tested as a dose of 0 05—1 0 μg which gave a blued area of a diameter from 3 mm to 10 mm Those limits of the size of the lesion cover the range of the linear response to bradykinin in experiments in guinea pig skin (Bhoola *et al* 1960)

Glandulain was tested as a dose of 0 5—7 0 μg A clear lesion (diameter about 4 mm) which could be differentiated from the traumatic blueing could be obtained only with doses over 5—7 μg Doses over 7 μg were not tested because of the dilution of the original enzyme preparation available

Kallikrein like peptidase The dose of the isoenzymes of this peptidase was chosen the same as with salivain A dose of 1 μg gave usually a lesion of 5 mm diameter The response to all of the isozymes (A B C and D) was about equal

Trypsin Trypsin is known to have a fairly low hypotensive effect in the rabbit and therefore considerably higher doses were used A response equal to that obtained with 0 3 μg of salivain was obtained with 25 μg of trypsin

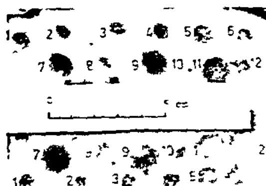


Fig 1 The lower part of the figure shows the response to the test substances before and the upper part 5 min after the injection of Phenergan ® 1 mg kg 1 = Kallikrein like peptidase isoenzyme A 1 μg 2 = Isoenzyme B 1 μg 3 = Isoenzyme C 1 μg 4 = Isoenzyme D 1 μg 5 = Trypsin 2 μg 6 = Histamine 5 μg 7 = Salivain 1 μg 8 = Glandulain 7 μg 9 = Depot Padutin ® 60 μg 10 = Saline 0 05 ml 11 = Bradykinin 10 μg 12 = Histamine 5 μg

Depot Padutin® This is a kallikrein preparation purified from hog pancreas and is known to liberate kinins One unit corresponding to about 60 μg of protein gave a lesion diameter 10—12 mm i e equal to or better than by 1 μg of Salivain

Bradykinin Twenty μg of bradykinin is known to give a lesion 6—7 mm by diameter in guinea pig (Bhoola *et al* 1960) The dose used by us as a standard was chosen as 10 μg and the lesion diameter was constantly 10—11 mm

Histamine This was tested as a dose 0 5—5 0 μg The lowest dose gave only a minimal response while 5 0 μg produced lesions equal to those obtained with the highest (used) doses of salivain Depot Padutin and bradykinin The lesion caused by histamine was qualitatively different from those produced by the other substances The lesions were paler and the distribution of the dye was not homogenous A pale center in the lesion was constantly found

Prometazine inhibited the lesion caused by histamine and also the traumatic lesion caused by injection but not that caused by the tested enzymes and by bradykinin The response to all of the enzymes tested was lowered to about one half by the prior injections of Butazolidin®

Discussion

Salivain seems to be the most potent one of the tested enzymes in increasing the vascular permeability This fits with the fact that salivain also has a very potent hypotensive effect The increase in the capillary permeability caused by the kallikrein like peptidase was marked but clearly weaker than that caused by glandulain This is what could be expected on the basis of earlier knowledge on the hypotensive and esterase activities of these enzymes (Ekfors *et al* 1967 a b) Although glandulain was without effect in the blood pressure test it now gave a positive result This difference is undoubtedly due to the different sensitivity of the methods The inhibition caused by prometazine excludes the possibility that the effect of the enzymes is mediated by histamine liberation Some characteristics of the lesions caused by the enzymes also resembled more those caused by bradykinin than those by histamine The histamine spots were more diffuse and a paler area could be seen in the middle of the lesion The lesions produced by bradykinin and by the enzymes were similar in that there was a greater accumulation of dye in the lesions and the dye was more evenly distributed

The partial inhibition of the effect of the enzymes on vascular permeability by phenylbutazone agrees with earlier findings The increased capillary permeability produced by various means has been shown to be diminished by phenylbutazone Voelkel 1956 Ratschow and Thure 1957 Northover and Subramanian (1961) when studying the effect of analgesic antipyretic drugs on kallikrein and plasmakinin formation could show that in rabbits the accumulation of protein bound dye at the site of injection of human salivary kallikrein and guinea pig serum kallikrein but not of bradykinin and kallidin was inhibited by the systemic administration of drugs including phenylbutazone On the ground of this finding they suggested the inhi

bition of kinin-forming enzymes to take place at a lower concentration of phenyl butazone than what is required to inhibit plasma kinin itself

These findings demonstrate that salivain and the kallikrein-like peptidase have a potent vascular permeability increasing effect and, to a lower degree, the same capacity is shared by glandulain These findings are in accord with those concerning the hypotensive effect of the same enzymes These effects are most likely mediated by the kinins liberated by the enzymes

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Circulatory and Respiratory Effects of Different Doses of Prostaglandin E_1 in Man

By

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Abstract

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At the higher dose levels there was a further increase in heart rate and decrease in brachial artery pressure The stroke volume decreased but the cardiac output remained above resting levels The high doses also produced an alveolar hyperventilation, a further increase in O_2 consumption and in three cases a respiratory alkalosis

Prostaglandins have been shown to cause a prolonged fall in blood pressure on i v injection in the rabbit and in the cat (Euler 1939) Bergstrom *et al* (1959) infused Prostaglandin E into human subjects and observed an increase in heart rate and a moderate fall in systemic arterial blood pressure and cardiac output In the last report only one concentration was used for each subject and the infusion was done over a short period (4—10 min) Bergstrom *et al* (1965) infused Prostaglandin E_1, (PGE_1), at a rate of 0 1—0 2 μg/kg/min into 3 healthy male subjects for 20 min and, besides the metabolic effects, observed that with this dose the brachial artery pressure was unchanged and the heart rate increased consistently When PGE_1 was infused together with noradrenaline, the pressure response to noradrenaline was reduced by approximately 50 per cent and the usual bradycardia was completely inhibited The present investigation was performed to further elucidate the effect of different doses of PGE_1 and to make a detailed examination of the cardiovascular reactions in man

Methods and Procedure

Procedure

8 healthy male volunteers aged 22—31 years (mean 26 9) were studied Prior to the experiment the subjects underwent examination of the circulatory system with recording of electrocardiogram at rest and during work, and testing of the working capacity (Sjostrand 1960) determined as work in

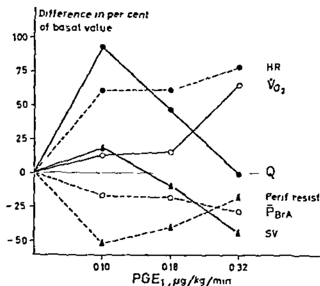


Fig 1 Circulatory data from subj 3 during continuous infusion of PGE$_1$ at increasing dose rates over 30 min periodes Data are given as a percentage difference between a 20 min value and the value obtained at rest Q = cardiac output The other symbols are the same as in Table I and II and III

29—93 per cent) in combination with a slight decrease in brachial artery mean pressure, mean 8 0 mm Hg (range 2—19), which indicated a decrease of the peripheral resistance (mean 41 1 per cent of resting value) The increased cardiac output was the result of a combined increase in heart rate (mean 27 3 beats/min) and in stroke volume (mean 21 7 per cent) The AVO. difference decreased on an average 29 1 per cent (range 15—42 per cent) The oxygen uptake also increased (mean 15 5 per cent) but the increase in cardiac output was much greater and not correlated to the oxygen uptake

With a higher dose of PGE$_1$ 0 18—0 32 μg/kg/min, the pulse rate increased further (mean 7 beats min) but the stroke volume returned to the resting value or became smaller which resulted in a decrease in cardiac output to values slightly higher than at rest (mean 30 8 per cent above basal) The mean arterial pressure decreased further (mean 9 mm Hg but with a smaller cardiac output the calculated peripheral resistance became somewhat greater but was still well below the resting value (mean 67 2 per cent of resting value)

The mean pressure in the pulmonary artery decreased on an average by 3 3 mm Hg (range 1—5) The systolic pressure did not change significantly The end diastolic pressure in the right ventricle decreased on an average by 3 3 mm Hg (range 2—4)

The arterial oxygen saturation (S_{aO_2}) decreased from an average of 97 6 per cent (range 96 4—99 2) at rest to an average of 96 1 per cent (range 94 2—98 3) during infusions at rates of 0 058—0 18 μg/kg/min S_{aO_2} then increased by, on an average, 1 65 per cent in 4 cases at higher infusion rates

The hemoglobin concentration increased in 5 of 6 subjects by 1 3—3 7 per cent from rest to the highest infusion level

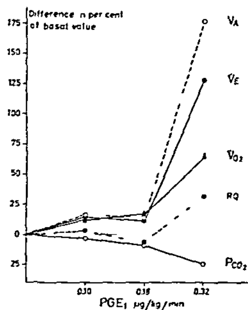
TABLE III Respiratory data at rest and during continuous infusion of PGE_1 at increasing dose rates over 30 min periods in 6 healthy young men V_E = total ventilation V_A = alveolar ventilation, V_{O_2} = oxygen uptake, RR = respiratory rate, RQ = respiratory quotient, P_{aCO_2} = carbon dioxide tension in arterial blood

Subj	Inf amount of PGE_1	V_E l/min BTPS	V_A l/min BTPS	V_{O_2} ml/min STPD	RR min^{-1}	RQ	P_{aCO_2} mm Hg	pH
3	Before	6 89	4 25	249	17	0 71	37	7 43
	0 10 μg/kg/min	7 92	4 90	281	16	0 73	36	7 46
	0 18 »	7 72	4 90	287	18	0 67	34	7 46
	0 32 »	15 8	11 80	411	16	0 94	28	7 49
	After	—	—	—	—	—	—	—
4	Before	6 74	4 20	260	14	0 69	37	7 41
	0 058 μg/kg/min	6 82	4 40	273	14	0 71	38	7 43
	0 10 »	8 11	5 30	268	12	0 84	37	7 41
	0 18 »	10 9	8 20	348	12	0 84	31	7 47
	0 32 »	20 5	16 2	450	21	0 95	23	7 55
	After	—	—	—	—	—	—	—
5	Before	7 81	4 55	277	17	0 70	37	7 42
	0 058 μg/kg/min	9 09	5 50	303	22	0 73	35	7 44
	0 10 »	11 4	8 60	331	19	0 88	29	7 48
	After	—	—	—	—	—	—	—
6	Before	10 5	6 75	294	16	0 75	31	7 44
	0 058 μg/kg/min	14 10	8 60	320	19	0 89	29	7 51
	0 10 »	15 0	9 50	327	17	0 86	26	7 54
	0 18 »	39 6	27 7	420	24	1 16	16	7 66
	After	—	—	—	—	—	—	—
8	Before	9 17	6 90	355	19	0 68	35	7 42
	0 058 μg/kg/min	10 1	7 90	336	14	0 73	35	7 42
	0 10 »	11 2	6 70	389	16	0 73	35 / 32	7 43 / 7 46
	After	—	—	—	—	—	—	—
9	Before	8 53	5 00	301	14	0 73	37	7 42
	0 058 μg/kg/min	9 19	5 60	299	14	0 77	36	7 44
	0 10 »	15 7	11 0	411	16	0 89	29	7 46
	0 18 »	21 5	16 0	512	20	0 93	25	7 50
	After	—	—	—	—	—	—	—

Respiratory response

For details see Table III and Fig 2

With a smaller dose of PGE_1, 0 058—0 10 μg/kg/min, the total ventilation (V_E) increased on an average 38 4 per cent (range 14 9—84 1) resulting in a slight alveolar hyperventilation in 3 of the subjects (cases 5, 6, 9), mean increase 23 6 per cent



[illegible] and the value obtained at rest. The symbols are the same as in Table III.

With a higher dose of PGE_1, 0.18—0.32 μg/kg/min, there was a marked further increase in total ventilation and also in the alveolar ventilation, the alveolar ventilation (V_A) increased further by on an average, 181.0 per cent (range 141—206) with a corresponding increase in total ventilation. The alveolar hyperventilation resulted in a respiratory alkalosis with P_{CO_2} values between 16—28 mm Hg and pH values between 7.49—7.66.

The oxygen uptake increased on an average by 15.5 per cent (range 3.1—36.5) at the smaller doses of PGE_1 0.058—0.10 μg/kg/min. At the higher doses of PGE_1 there was a marked further increase of oxygen uptake on an average by 41.8 per cent (range 24.6—67.9).

The overall ventilation perfusion relationship of the lungs (V_A/Q) decreased from a basal mean value of 0.72 to a mean of 0.56 at dose rates of 0.058—0.10 μg/kg/min. At the higher doses the quotient increased to a mean value of 1.93.

The plasma renin activity was normal at rest before the infusion (mean value 129.8 ng angiotensin/100 ml plasma, range 64—237), increased in two subjects and remained unchanged in two.

Discussion

The increase in pulse rate at the higher dose levels was of similar magnitude to that found by Bergstrom *et al.* (1959). With the lower doses, especially in cases 1 and 2, there was an increase in pulse rate without change or with only a slight increase in arterial blood pressure, as was also seen by Bergstrom *et al.* (1965). This suggests that the increase in pulse rate is not due to a baroreceptor reflex stimulation induced

by a blood pressure fall but rather to stimulation of the sympathetic nervous system (Carlson and Oro 1966) or a direct effect on the heart Nakano and McCurdy (1967) also found a marked increase in heart rate after injection of PGE_1 into dogs and interpreted it as a result of a reflex sympathetic stimulation The findings of Bergstrom *et al* (1965) that the bradycardia induced by a noradrenaline infusion was abolished by a simultaneous infusion of PGE_1 also support the theory of a direct action of PGE_1 on the heart

The marked increase in cardiac output found in the present study at the lower dose levels was caused both by an increase in stroke volume and by an increased heart rate An increase in heart rate will in itself not necessesarily produce an increase in stroke volume as, for instance, methylscopolamine increases the heart rate markedly but decreases stroke volume, so that the cardiac output is unchanged (Bevegård 1963) In the present study, in addition to the marked rise in heart rate, there was a marked fall in the systemic resistance, which increases the kinetic filling energy and decreases the resistance to emptying of the heart This certainly contributes to the increase in stroke volume There is thus a hyperkinetic circulation with a large cardiac output in relation to the oxygen uptake and a low AVO_2 difference At higher dose levels there was a further rise in heart rate and fall in brachial artery mean pressure but the stroke volume decreased, so that the cardiac output fell to the same level as at rest or slightly above that level This may partly be due to the successive shortening of diastole with increasing heart rates which, in combination with a decrease in the available filling energy (a change in the capacity vessels with an increase of total capacity), will result in a smaller stroke volume than at the lower dose levels

The systolic pressure in the right ventricle was on an average unchanged but the end diastolic pressure decreased in spite of a marked growth of stroke volume, indicating a change in the myocardial function curve This theory is supported by the results from experiments on dogs reported by Nakano and McCurdy (1967) They found that i v injected PGE_1 had a positive inotropic effect with an increased rate of rise of isometric tension (dp/dt) for the left ventricle and an increased myocardial contractile force for both ventricles A β blocking agent did not block either this positive inotropic effect or the strong vasodilator action of PGE_1 The mean pressure in the pulmonary artery decreased slightly and so did the diastolic pressure in the pulmonary artery, which corresponds to the pulmonary wedge pressure so that the pulmonary resistance decreased as much as the flow increased This change in pulmonary resistance is probably the result of a passive dilatation due to the increased flow

A hyperkinetic circulation is present in congenital and acquired A—V shunts This type of circulation has also been observed by Castenfors *et al* (1962) in circulatory studies on patients with dumping syndrome These cases showed an A—V shunting of blood through the splanchnic organs with a markedly increased splanchnic blood flow In a separate study with infusion into the femoral artery Carlson Ekelund and Oro (1968 found a roughly 10-fold increase in leg flow at a dose rate

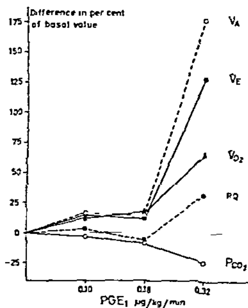


Fig 2 Respiratory data from subj 3 during continuous infusion of PGE_1 at increasing dose rates over 30 min periods Data are given as a percentage difference between a 20 min value and the value obtained at rest. The symbols are the same as in Table III

With a higher dose of PGE_1, 0 18—0 32 µg/kg/min, there was a marked further increase in total ventilation and also in the alveolar ventilation, the alveolar ventilation (V_A) increased further by on an average, 181 0 per cent (range 141—206) with a corresponding increase in total ventilation The alveolar hyperventilation resulted in a respiratory alkalosis with P_{CO_2} values between 16—28 mm Hg and pH values between 7 49—7 66

The oxygen uptake increased on an average by 15 5 per cent (range 3 1—36 5) at the smaller doses of PGE_1, 0 058—0 10 µg/kg min At the higher doses of PGE_1 there was a marked further increase of oxygen uptake, on an average by 41 8 per cent (range 24 6—67 9

The overall ventilation-perfusion relationship of the lungs (V_A Q) decreased from a basal mean value of 0 72 to a mean of 0 56 at dose rates of 0 058—0 10 µg kg/min At the higher doses the quotient increased to a mean value of 1 93

The plasma renin activity was normal at rest before the infusion, mean value 129 8 *ng angiotensin 100 ml plasma* (range *64—237*), increased in two subjects and remained unchanged in two

Discussion

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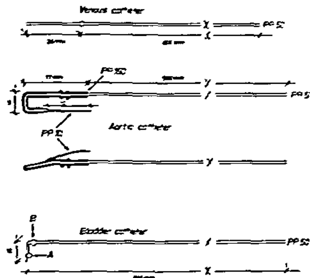


Fig. 1 Polypropylene catheters.

used for anchoring the catheter (see below) but during fashioning of the bends for protecting the interior P.P. 50.

The large size — P.P. 50 — of the venous catheter is preferred to permit monitoring of central venous pressure.

Surgical procedure

Rats weighing 200—300 g are used. As anaesthetic, ether on open mask is preferred, mainly because of the quick postanaesthetic recovery. Surgical technique is clean but not sterile. All catheters are filled with saline prior to their implantation and afterwards sealed by heating. Heparin is not used.

After induction of anaesthesia, a small incision is made in the skin between the scapulae. The animal is then turned on its back and the ventral part of the neck and the abdomen is shaved and covered with Nobecutan®[1].

The right or left jugular vein is exposed through a small vertical incision immediately above the clavicle. The vessel is raised with a pair of small forceps, ligated distally and incised. The introduction of a catheter is facilitated by lifting the ventral vein wall with a small right angled hook (manufactured from a 12 gauge needle). A part of the catheter up to and including the expansion is inserted and a ligature is tied behind the expansion. The tip of the catheter should now be immediately above the right atrium. This is verified by gentle aspiration with a syringe. The catheter is thereafter filled with saline. By blunt subcutaneous dissection, the sealed catheter is brought out through the prepared incision between the scapulae and the neck incision is closed with 4-0 silk.

The abdomen is then opened through a [illegible]0—60 mm long incision in the linea alba. The bulk of the intestines is lifted out to the left and covered with moist gauze. After identification of the ureters, the aorta is exposed by sharp dissection 10—12 mm proximally from the bifurcation. With the help of a smoothly tipped trochar (220 mm long, 2.0 mm diameter) an aortic catheter is brought out through the left psoas muscle 4—5 mm above and 3—4 mm lateral to the aortic bifurcation and between the genito-femoral nerve and the ureter, and then subcutaneously through the interscapular incision. The aorta is gently lifted up with a pair of smooth forceps and clamped with a modified vascular clamp[2] (Fig. 2a). Part A of the clamp should be underneath the vessel. With a sharp 20 gauge needle, the aorta is punctured 3—4 mm above the bifurcation. The tip of the aortic catheter is introduced through the hole in the aortic wall, and after releasing the clamp advanced into the junction between the P.P. 10 and the P.P. 50. If the position is correct, blood should immediately flush through the catheter. The catheter is filled with saline and sealed. Bleeding is usually minimal and easily controlled by direct gentle pressure for 2—3 min. The catheter is

[1] Bofors AB, Karlskoga, Sweden.

[2] Manufactured from an ordinary vascular clamp ([illegible] SERREFINE, 1 1/4 inches, curved), The Lawton Company Inc., 425 4th Ave., New York [illegible], U.S.A.

Acta physiol. scand. 1969. 75. 170—175

From the Department of Surgery, University Hospital, and Research Division, Pharmacia AB, Uppsala, Sweden

A Technique for Repeated Renal Clearance Measurements in Undisturbed Rats

By

ANDERS ENGBERG[1]

Received 13 May 1968

Abstract

ENGBERG, A *A technique for repeated renal clearance measurements in undisturbed rats* Acta physiol scand 1969 *75* 170—175

A technique for simultaneous i v infusion into and blood and urine sampling from relatively unrestrained rats Detailed description of the design of catheters, of their implantation and of sampling procedures Control autopsy results are discussed

es on renal function often necessitate single or repeated experiments on animals ig for several hours If possible, these experiments should be performed without aesthesia, as the anaesthetic may obscure the results

The advent of modern plastic materials has made possible the use of long term implanted catheters for painless sampling Dogs, e g , can be trained to accept the experimental conditions, but as regards several other species any restraint imposed may have adverse effects In rats, restraint alone more than doubles the cortisone excretion (Knigge *et al* 1959) and it also causes an increased gastric acidity (Brodie *et al* 1962) Reports on renal function in unanaesthetized rats (Cotlove 1961, Berglund 1962, Malvin 1967) usually involve some form of restraint In 1965 Kleinman *et al* , using implanted catheters, described a method of studying renal clearances in undisturbed rats The technique to be described is basically similar, but there are some important modifications in catheter design, surgical procedure, sampling technique, and in the care of the animals

Material and methods

Catheter design

[illegible] excellent description [illegible]

[1] Present address Department of Surgery University Hospital, Uppsala, Sweden

[2] Portex Ltd , England (Numbers refer to their scale)

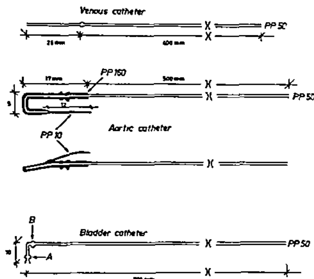


Fig 1 Polypropylene catheters

used for anchoring the catheter (see below) but during fashioning of the bends for protecting the interior P P 50

The large size — P P 50 — of the venous catheter is preferred to permit monitoring of central venous pressure

Surgical procedures

Rats weighing 200—350 g are used As anaesthetic, ether on open mask is preferred, mainly because of the quick postanaesthetic recovery Surgical technique is clean but not sterile All catheters are filled with saline prior to their implantation and afterwards sealed by heating Heparin is not used

After

[1] Bofors AB, Karlskoga Sweden
[2] Manufactured from an ordinary vascular clamp (GL-665 SERREFINE, 2 1/4 inches curved) The Lawton Company Inc 425 4th Ave New York 16 U S A

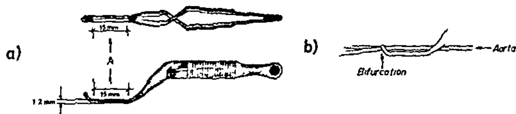


Fig 2 a) Vascular clamp
b) Clamp on the abdominal aorta

anchored in the psoas muscle with a 4 0 silk ligature loosely tied between the P P 160 expansions The posterior peritoneum and the intestines are replaced

The troachar is brought subcutaneously from the lowest part of the abdominal incision past the left flank to the interscapular incision, and a bladder catheter is inserted to be ready for implanta-

the abdominal muscle suture, which is tied around the tube above the proximal expansion (B) The abdominal incision is closed in 2 layers with continuous 4 0 silk and covered with Nobecutan To avoid later contamination of urine samples, the tail is amputated using a fish mouth incision of the skin, leaving a stump of 35—40 mm

The animal is turned on to its abdomen and the catheters are assembled with the help of adhesive tape The catheter assembly is taken through an opening in the bottom of a small light weight plastic hollow cap (Fig 3 A) This cap is sutured (3-0 twisted steel wire) in 4 places to the skin

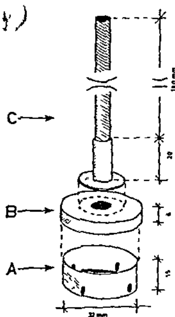


Fig 3

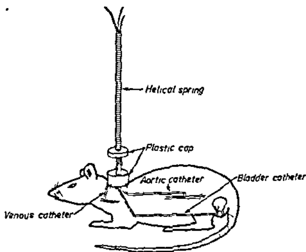


Fig 4

Fig 3 Plastic hollow cap (A), plastic cover (B) and helix spring (C)
Fig 4 Position of catheters

¹ Ethicon Ltd , Hamburg-Glashutte, Germany

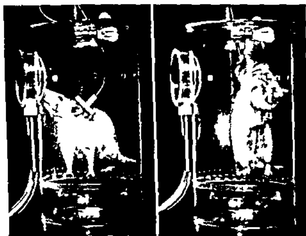


Fig 2 Rat in metabolic cage

around the interscapular incision The catheters are coiled in the cap and protected by a ...
soft plastic cover (Fig 3 B) The animals are
their preoperative activity and food intak
are not flushed between experiments Fig

Sampling and infusion procedures

another, to which is attached a helix spring (Fig 3 B+C) The catheter assembly is passed through the spring which prevents the animals from damaging the catheters The helix spring is suspended from a swivel This arrangement permits the animal to move around freely (Fig 5) It is now possible to infuse through the venous catheter and to take repeated blood samples from the aortic catheter without disturbing the animal If necessary the catheters are easily cleared by forceful injection of 0 05 ml of saline Between sampling the catheter is filled with saline

Most of the urine is collected by permitting the animals to void spontaneously To ensure a complete collection 1 8 ml saline is injected into the bladder with an automatic calibrated syringe This procedure is repeated 2—3 times, and the animals void spontaneously at each saline injection After the saline washing 5 ml of air are slowly injected through the bladder catheter in order to induce a complete bladder evacuation

Results and conclusions

This report is based on 150 rats A detailed control autopsy study has been performed on 116 animals (Table I) The procedures were modified during the course of the experiments but the modifications reported here were used in the last 70 consecutive animals The operative mortality has been less than 3 per cent

TABLE I

Time from catheter implantation to autopsy	<1 week	1—2 weeks	2—3 weeks	>3 weeks	Total
Number of rats	17	48	17	34	116
Thrombus around venous catheter	3	12	7	11	33
Thrombus in distal aorta	—	3	—	3	6
Hydronephrosis left	3	2	1	3	9
Kidney infarction	1	1	3	7	12
Bladder stones	1	5	3	19	28

Function of catheters

The majority of the experiments were performed within 3 weeks after the implantations of the catheters. Except in very rare instances, the venous and aortic catheters were patent. In the first 46 animals, I used a bladder catheter with a simple flange at the tip as described by Kleinman *et al.* (1965). *These catheters were fairly often blocked by the bladder wall or dislocated. It was also difficult to achieve complete* emptying of the bladder repeatedly (tested by placing known volumes of fluid ng a colour indicator, a blue dextran, in the bladder). The bladder catheter then modified as described and has since functioned satisfactorily. Kleinman *et al.* (1965) recommended 3 ml of saline for washing the bladder. In my experience this volume is too large as it very often causes haematuria.

Autopsy results

The relevant autopsy data are summarized in Table I. Infections were extremely rare. The frequency of thrombosis around the venous catheter was high. (However if the catheter is properly positioned with the tip in the upper part of the right atrium, it functions in spite of the thrombosis.) As rats have two superior vena cava the thrombosis has minimal effects on the venous return. As for the aortic complications, they were all caused by difficulties in introducing the catheter. Hydronephrosis was moderate and was generally caused by kinking of the left ureter by an incorrectly fashioned aortic catheter. Infarctions of the kidneys increased in frequency with time, but occurred less frequently when the P.P. 10 part was shortened to 12 mm. Infarctions of the kidneys were presumably caused by small emboli originating from the tip of the aortic catheter.

The frequency of bladder stones was reduced after the modification of the bladder catheter and the collection procedure. (Bladder stones are, however, still common 3—4 weeks after the catheter implantation. Hence the animals should be used within the first 3 weeks.)

In the first 52 rats of this series the catheter assembly was protected by a helix spring sutured to the animal's neck as devised by Kleinman *et al.* (1965). In spite

of different arrangements for supporting these springs, the animals failed to thrive With the cap technique, the body weight increase has been the same as for control animals

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Acta physiol. scand. 1969. 75. 176—186

From the Department of Physiology, University of Göteborg, Sweden

Hypothalamic Control of Adrenergic Outflow to the Stomach in the Cat

By

G JANSSON, B LISANDER and J. MARTINSON

Received 16 May 1968

Abstract

JANSSON, G, B LISANDER and J MARTINSON *Hypothalamic control of adrenergic outflow to the stomach in the cat* Acta physiol scand 1969 *75* 176—186

Experiments were performed on chloralosed, adrenalectomized cats with recording of gastric volume, blood pressure and skeletal muscle blood flow The vagal nerves were cut, but the vagal excitatory fibres to the stomach could be activated by electric stimulation Topical stimulation of the hypothalamic defence area or surrounding 'pressor' areas produced not only the characteristic known circulatory responses, but also a prompt and often complete inhibition of a vagally induced increase of gastric motility —But when the stomach was *not* under the influence of continuous vagal excitatory activity corresponding stimulation of the hypothalamus rarely had any effect on gastric volume and then only an insignificant and sluggish increase despite considerable myogenic tone of the stomach Stimulation of the adjacent hypothalamic sympatho-inhibitory area on the other hand, augmented the vagally induced gastric motility and thereby suggested the presence of a centrally induced suppression of a prevailing sympathetic inhibitory influence on the stomach —All the above mentioned effects on gastric volume, produced by central nervous stimulation could be blocked by guanethidine in these vagotomized cats It is concluded that the hypothalamus contains neural mechanisms which can affect stomach motility by changing in both directions the impulse frequency in the inhibitory adrenergic outflow to this organ Simultaneous activity of vagal excitatory fibres to the stomach was found to be a prerequisite for reducing significantly gastric motility and tone when the adrenergic fibres were excited This is compatible with the hypothesis that the adrenergic fibres exert their inhibitory effect mainly or only by acting on the parasympathetic ganglionic cells, which mediate the vagal excitatory influence to the gastric smooth muscles

The vagal nerves contain two types of efferent fibres to the stomach One group elicits an excitatory, atropine-sensitive motor response the other, a profound relaxation of the corpus-fundus section of the stomach (Jansson and Martinson 1965) The latter response is resistant to atropine and antiadrenergic drugs (Martinson 1965)

Furthermore, the intestino-gastric inhibitory reflex (IGIR), which acts by an efferent adrenergic mechanism *via* the splanchnic nerves, seems to exert its action on

the stomach preferentially, or only, by inhibiting parasympathetic ganglionic cells involved in the excitatory control of gastric motility (Jansson and Martinson 1966) Thus, in the acutely vagotomized cat this reflex did not significantly reduce gastric tone, which in this situation is held to be myogenous with little or no cholinergic activity present with respect to motility control

However, it cannot be excluded *a priori* that other types of activation of the adrenergic nerve supply to the stomach can also involve fibres in direct contact with the gastric smooth muscles At any rate, the possible presence of a sympathetic inhibitory action on the intramural parasympathetic ganglionic cells calls for reconsideration of the mode of action of the central nervous control of gastric motility in preparations where the 'background vagal excitatory activity can be controlled

In many earlier investigations the central nervous control of gastric motility has been studied in preparations where the entire nervous supply to the stomach has been left intact and where, therefore, the degree of activity of the vagal excitatory fibres was not known (for ref see Kaada 1951—52, Eliasson 1952) It is, then, obviously difficult to find out whether *e g* an inhibitory response is due to an activation of the vagal 'relaxatory' fibres, to an inhibition of existing activity of vagal excitatory fibres or to an activation of sympathetic fibres that suppresses the effect of tonically active vagal excitatory fibres Conversely, if a centrally induced enhancement of gastric tone is eliminated by section of the sympathetic fibres, it does not necessarily imply that sympathetic excitatory fibres are present Such a response may equally well be due to a central inhibition of a prevailing sympathetic activity with suppression of the effect of tonically active vagal excitatory fibres to the stomach

The hypothalamus is known to influence most autonomic functions often inducing changes integrated to well-defined response patterns Thus topical stimulation of the hypothalamic defence area can induce a differentiated pattern of sympathetic activity well suited for emergency situations and believed to occur in states of fear rage or arousal (for ref see Abrahams Hilton and Zbrozyna 1960) —Stimulation of adjacent areas elicits responses, for convenience called pressor responses which can induce a more or less general increase of the adrenergic effects on the cardiovascular system without involving the active cholinergic vasodilatation typical of the defence reaction (see *e g* Feigl 1964) —Further stimulation of the hypothalamic sympatho-inhibitory area (Folkow, Johansson and Öberg 1959) also adjacent to the defence area, results in a pronounced inhibition of sympathetic tone in the entire cardiovascular system combined with vagal inhibition of the heart

In view of the implications of the investigation by Jansson and Martinson (1966) and the considerations outlined above it was considered legitimate to try to find out how the adrenergic outflow to the stomach is influenced by topical stimulation of the above mentioned hypothalamic areas and how they interact with the vagal excitatory control of the stomach in preparations with a known level of activity of the vagal excitatory fibres Preliminary results of this study have been briefly reported elsewhere Jansson, Lisander and Martinson 1966)

Material and methods

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was ligated at the cardia, care being taken not to damage the vagal trunks A wide bore plastic catheter was introduced int
containing isotonic saline, ke
volume by a float recorder
The duodenum was dividec
introduced into the lumen to allow free drainage of bile and pancreatic secretion The rest of the intestinal tract was extirpated

Arterial blood pressure was continuously recorded by means of a mercury manometer connected to a cannula inserted into one of the femoral arteries Skeletal muscle blood flow was determined as the outflow from the femoral vein, the paw circulation being excluded by a tight ligature The blood was conducted through a photo-electric drop chamber operating an ordinate writer and returned to the animal via the contralateral femoral vein In some experiments the calf muscles were isolated (Kjellmer 1964) in order to obtain a more accurate measurement of the muscle blood flow

The vagal nerves were dissected and divided in the neck The peripheral ends of both nerves were placed in annular bipolar silver electrodes and connected to square wave impulse generators (Grass Stimulator S4) Stimulations of 1—8 imp/sec at a voltage of 4—6 and a pulse duration of 0 5—2 msec were employed

For hypothalamic stimulation the head of the animal was immobilized in a Horsley Clarke

and then with 10 per cent formalin, and sectioned with a stereotaxically mounted knife The

To eliminate disturbing respiratory movements or reflex movements of the skeletal muscles, the animals were often curarized with gallamoni jodidum (Flaxedil®, May & Baker) in amounts of 2—4 mg/kg b w

Other drugs were administered i v atropine in a dose of 0 1—0 5 mg/kg b w (Atropine sulphate, Merck), phenoxibenzamine in a dose of 2—5 mg/kg (Dibenzyline®, Smith, Kline & French) and guanethidine (Ismeline® CIBA 3—5 mg/kg)

Results

I Comparison between the intestino-gastric inhibitory reflex and inhibition of gastric motility induced by hypothalamic stimulation Distension of an intestinal loop or electric stimulation of afferent mesenteric nerves inhibits vagally elicited contractions of smooth muscle cells in the stomach wall (cf Jansson and Martinson 1966) Fig 1 (left panel) illustrates such an experiment in which an elicitation of the intestino-gastric inhibitory reflex completely inhibits the vagally induced excitatory motility A similar inhibition can be induced by stimulation of a hypothalamic "pressor" area (Fig 1 right panel) When the vagi were not stimulated in the acutely vagotomized cat, the stomach was considered to be in a state of basal tone (cf Jansson and Martinson 1966) and then adrenergic activation of this type had no or little inhibitory effect, and, if any, one with a comparatively long latency The inhibitory responses (Fig 1) did not extend beyond the basal tone state

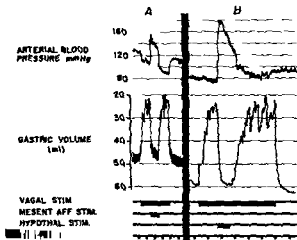


Fig 1 Comparison between the inhibitory response of the intestino-gastric inhibitory reflex (A) and inhibition of stomach motility upon stimulation of a hypothalamic "pressor" area (B)
A Cat 2 4 kg Cervical vagal stimulation 4 imp/sec 2 msec, 5 V Mesenteric afferent nerve stimulation 4 imp sec 2 msec 3 V
B Cat 2 9 kg Cervical vagal *stimulation 4 imp/sec, 2 msec, 5* V Hypothalamic stimulation (90 imp/sec 1 5 msec 4 V) with pressor *response and the stimulation point* is illustrated in Fig 6 (left panel) as the filled circle in the electrode track

2 *The defence reaction and inhibition of gastric motility* Defence reactions were regularly elicited by stimulation of a hypothalamic area corresponding to that outlined by Abrahams, Hilton and Zbrozyna (1960) Fig 2 shows such a cardiovascular response with the characteristic cholinergic vasodilatation in skeletal muscle and a slight increase in blood pressure Vagally induced gastric motility was inhibited as part of this reaction In the experiment illustrated in Fig 5 there is virtually complete elimination of the vagally induced excitatory response during stimulation of the defence area

3 *Hypothalamic pressor responses together with gastric motor inhibitions* Fig 3 shows the effect, on gastric motility, of electric stimulation during stepwise lowering of the

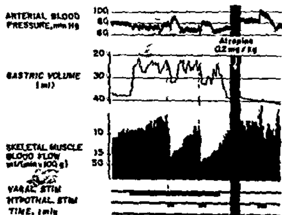
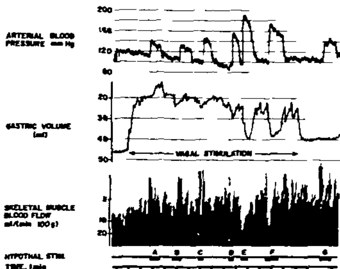


Fig 2 Cat 2 7 kg Stimulation of defence area (80 imp sec 2 msec 6 V with inhibition of vagally induced excitatory stomach motility and the characteristic circulatory adjustments Cervical vagal efferent stimulation 4 imp sec 2 msec 5 V *Right panel* After administration of atropine stimulation of the defence area had *no effect on gastric tone* Observe the complete disappearance of the vasodilatation in skeletal *muscle The site of the stimulation* point is given in Fig 6 (hollow circle below electrode track in left panel



stimulation electrode in the hypothalamus. Varying pressor responses were obtained together with a varying degree of inhibition of the gastric motility elicited by vagal stimulation except in point A where a slight augmentation of gastric tone was noted. Although the skeletal muscle blood flow obviously increased during stimulation E and F flow resistance in this vascular bed was not decreased by more than what could be explained by activation of the baroreceptors by the marked rise in blood pressure. Note the parallelism between the magnitude of the blood pressure response and gastric motor inhibition during stimulation from B to F. It is clear from the curves that the inhibitory responses never extended beyond the level of basal tone of the stomach. Pressor responses of this type could be evoked from very widespread areas in the hypothalamus but they were more commonly encountered and more pronounced on stimulation in the vicinity of the defence area.

4 Hypothalamic sympatho inhibitory responses and augmentation of gastric motility. Sympatho-inhibitory responses with respect to the cardiovascular system could be obtained by electric stimulation of a small area close to the anterior commissure and about 2 mm from the midline. The "sympatho-inhibitory area" (Folkow, Johansson and Öberg 1959). Simultaneously with the known circulatory adjustments there was an augmentation of the vagally induced excitatory motility (Fig. 4 and 5). However, no augmentation of gastric tone was obtained upon stimulation of the hypothalamic sympatho-inhibitory area unless also the vagal nerves were stimulated (Fig. 5).

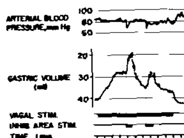


Fig 4 Cat 3 3 kg Augmentatory gastric motor responses on stimulation of the sympatho-inhibitory area in hypothalamus The site of the stimulation point is given in Fig 6 (triangle in left panel) Vagal stimulation 2 imp/sec, 2 msec, 5 V Hypothalamic stimulation 80 imp/sec, 1 7 msec, 4 V

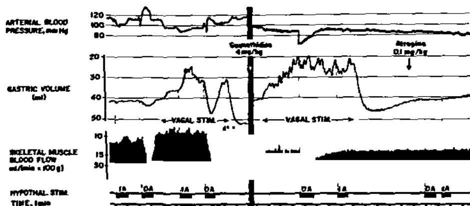


Fig 5 Cat 4 0 kg Adrenalectomized and vagotomized Inhibition and augmentation of vagally induced stomach motility on stimulation of hypothalamic defence area (DA) and sympatho-inhibitory area (IA) (60 imp sec, 1 msec, 4 V) The sites of the stimulated points are given on the right panel in Fig 6

Left panel Stimulation of sympatho-inhibitory area without simultaneous vagal stimulation had no effect on actual gastric tone and stimulation of defence area elicited a faint volume increase with a comparatively long latency besides the circulatory responses During continuous vagal stimulation (6 imp sec 2 msec 5 V) the stimulation of the sympatho-inhibitory area elicited a marked augmentation of the stomach motility while stimulation of the defence area promptly and effectively inhibited the motor response

Right panel After administration of antiadrenergic drug (guanethidine) stimulation of the hypothalamus had no effect on vagally induced motility Observe the more pronounced excitatory motor response to the same vagal stimulation and the persisting vasodilatation on stimulation of the defence area After administration of guanethidine and atropine stimulation of the hypothalamus produced no change in the circulation or in gastric myogenic tone

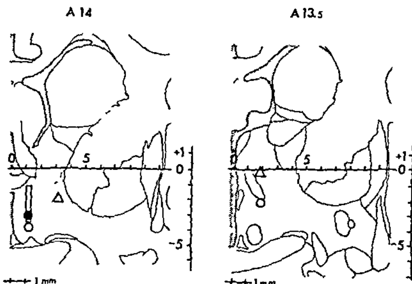


Fig 6 Frontal sections of the cat's brain illustrating the histologically verified sites of stimulatic.. points from where the responses in the previous figures were obtained
Left panel (A 14) The filled circle shows the location of the pressor response point where stimulation

stomach motility

5 *Assessment of the adrenergic character of the hypothalamic gastric responses* To minimize possible local circulatory changes in the stomach wall during the hypothalamic stimulations in these experiments, in some experiments the a-blocking agent phenoxibenzamine was administered in a dose of 2—5 mg/kg b w Hypothalamic pressor responses were then abolished or markedly reduced, being now dependent only on an increase in heart activity, and the defence reactions were associated with a fall in blood pressure due to the cholinergic vasodilatation However, the inhibition of the stomach was just as pronounced as before administration of the drug, indicating that local circulatory changes can have no appreciable effect on the gastric inhibition Administration of guanethidine, on the other hand, completely abolished the gastric effects of stimulation of the defence area and the sympatho-inhibitory area, while the cholinergic muscle vasodilatation persisted and the vagal excitatory response was enhanced

Fig 5 illustrates an experiment in which the defence reaction and the sympatho-inhibitory reaction were induced by hypothalamic stimulation within the same electrode track with a stepwise moved electrode A ventral shift of the electrode tip by 2 mm changed the inhibitory reaction into a defence reaction This figure also

shows that stimulation of the inhibitory area had no effect on the actual gastric tone in the absence of a continuous background vagal stimulation and that stimulation of the defence area produced only a slight motor inhibition with a comparatively long latency When, on the other hand, these stimulations were repeated during a period of continuous vagal excitatory stimulation, the inhibitory response was accompanied by a marked enhancement of gastric tone and the defence reaction by a profound inhibition of gastric tone Administration of the antiadrenergic drug, guanethidine, abolished the inhibitory effect of stimulation of the defence area as well as the augmentatory motor response during stimulation of the inhibitory area The cholinergic vasodilatation in skeletal muscle persisted during stimulation of the defence area and thereby confirmed the 'functional localization" of this hypothalamic area

Discussion

The experiments showed that hypothalamic structures can regulate stomach motility by changing the activity in the adrenergic neurons to this organ This is in accord with the conventional view that excitation of adrenergic nerves will inhibit gastrointestinal motility However, when studying the intestino gastric inhibitory reflex Jansson and Martinson (1966) produced evidence that the efferent adrenergic link in this reflex does not act directly on the gastric smooth muscle cells but at the level of the cholinergic ganglionic plexa in the wall, and thereby suppresses the trans mission of impulses by the vagal excitatory fibres Observations made in the present investigation suggest that the hypothalamus exerts its influence on gastric tone by a similar peripheral mechanism

The present experiments further reveal that stimulation of the hypothalamic sympatho inhibitory area augments the motor effect of the vagal excitatory fibres, but does not affect gastric myogenic tone (*cf* Jansson and Martinson 1966) This indicates that the above mentioned hypothalamic area acts by suppressing an adrenergic "background' discharge Furthermore, the effect is abolished by guan ethidine which, if anything, augments cholinergic effects suggesting that the augmented gastric motility depends on a decreased tonic adrenergic discharge to the stomach Under the present experimental conditions, which include abdominal surgery, there is a background adrenergic reflex discharge which inhibits the cholin ergic excitatory system controlled by the vagal nerves Suppression of this adrenergic discharge will result in a release of vagal excitatory influence on the stomach smooth muscle cells with augmentation of the vagal excitatory motor response

If the nervous supply to the stomach is intact, a varying and rather uncontrolled reflex and central nervous tonic discharge mediated *via* the vagal and splanchnic nerves affect stomach motility to an extent varying with the experimental conditions Demonstration of the above mentioned inhibition and augmentation of stomach motility requires either a vagal tonic discharge or combined vagal and sympathetic tonic discharge, respectively These findings have at least one important implication,

viz they question the validity of conclusions by earlier investigators who tried to trace the efferent pathways of central nervous control of gastric motility by cutting different nerves to the stomach For instance, if an inhibitory response was abolished by cutting the vagi, this could, of course, mean that the response was due to an inhibition of prevailing vagal excitatory activity, but it might equally well have been of sympathetic inhibitory origin, since some degree of "background" excitatory vagal activity is a prerequisite for revealing changes in activity of sympathetic neurons to the stomach. For the same reason, atropine can mask the activity of the adrenergic innervation of the stomach and thereby introduce another possible source of error in identifying the route of the efferent pathways to the stomach and the transmittor mechanisms involved Some of the findings obtained by Beattie and Sheehan (1934), Babkin and Kite (1950), Eliasson (1952, 1953—54) and Hesser and Perret (1960) must therefore be re-evaluated in experiments using various known degrees of the "background" activity of the vagal excitatory fibres

There is increasing evidence suggesting an important supraspinal control of the transmission in different somatomotor (*e g* Holmqvist and Lundberg 1962) and autonomic reflex paths (Johansson, Jonsson and Ljung 1965) It has been suggested that the regulating mechanisms from higher centres should operate to a large extent by their action on the interneurones of the spinal reflex arcs It is probable, for instance, that the hypothalamus exerts its above described influence on stomach otility by facilitating or suppressing the adrenergic intestino-gastric inhibitory reflex, though the present experiments were not directly aimed at proving the existence of such a mechanism The present findings are compatible with such an arrangement It deserves to be mentioned that Johansson Jonsson and Ljung (1965) showed that the intestino-intestinal inhibitory reflex was suppressed by stimulation of medullary depressor areas The similarity to the effect of stimulation of the hypothalamic sympatho inhibitory area is obvious

In the present experiments the changes in gastric motility produced by hypo thalamic stimulation, were often accompanied by pronounced circulatory changes The question then arises whether an alteration of the gastric blood flow can affect gastric motility Profound changes in gastrointestinal circulation can no doubt be elicited by such hypothalamic stimulation as that used in the present experiments (Cobbold *et al* 1964) But since the latency of the present gastric motor responses was very short, and since an α adrenergic blocking agent in amounts large enough to minimize vasoconstrictor fibre effects did not block the changes in gastric motility, the possibility that the motor effects should be due to changes in the regional blood supply can apparently be ruled out Furthermore, the fact that changes in gastric tone were negligible in the absence of vagal excitatory motor activity is incompatible with such a possibility

Nevertheless, the question arises whether the sympathetic effect on stomach motility, elicited by hypothalamic topical stimulation, is also normally associated with the characteristic circulatory adjustments, and thereby forming generalized sympathetic response patterns The present experiments indicate that this is as a

rule, the case, though not all hypothalamic pressor responses were accompanied by signs of an increased sympathetic discharge to the stomach But inhibition of stomach motility was invariably stronger when the pressor responses were more pronounced and especially in association with the defence reaction On the other hand, slight pressor responses sometimes occurred without any appreciable changes in gastric motility (Fig 3) Stimulation A in this figure also shows the unusual combination of a pressor response and a rise in gastric tone This finding may be due to overlapping of adjacent neuron pools, normally involved in separate activity patterns, but it may also be due to a secondary baroreceptor stimulation with a consequent reflex suppression of the inhibitory adrenergic discharge to the stomach

The hypothalamus is generally considered to be important for integrating the efferent expressions associated with emotion and it is a common experience that states of fear and anger are accompanied by powerful autonomic reactions The hypothalamically elicited defence reaction, which closely mimicks autonomic changes observed in the unanaesthetized animal showing behavioural signs of fear or rage is probably the integrative station for producing *e g* the well known cardiovascular adjustments in alarm situations (*e g* Abrahams, Hilton and Zbrozyna 1960) The present study showed that these circulatory changes are accompanied by marked changes in gastric motility It is of interest to recall the observations by Wolf and Wolff (1943) in their fistulous subject, Tom, *i e* emotions involving a desire for escape were accompanied by a depression of gastric function reflected in mucosal blanching, reduced acid production and inhibited motor activity

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Acta physiol scand 1969, 75 187—198

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Blockade of the Phosphorylase *a* Activating Effects of Catecholamines and Adenosine 3',5'-Monophosphate (Cyclic AMP) in High K^+ or Choline Buffer Media

By

Lennart Lundholm, Ella Mohme-Lundholm and Nandor Vamos

Received 22 May 1968

Abstract

Lundholm, L., E Mohme Lundholm and N Vamos *Blockade of the phosphorylase a activating effects of catecholamines and adenosine 3',5' monophosphate (cyclic AMP) in high K or choline buffer media* Acta physiol scand 1969 75 187—198

In isolated rat diaphragm suspended in a buffer solution where Na had been exchanged for K or choline the basal phosphorylase *a* activity was reduced and the phosphorylase *a* ac [illegible]

Addition of cyclic AMP to the buffer solution had a stimulating effect on the phosphorylase *a* activity of the rat diaphragm This effect was blocked or reduced when Na in the buffer [illegible]

Catecholamines have been shown to stimulate the production of cyclic AMP and thereby activate phosphorylase (Sutherland and Rall 1960) Cyclic AMP is formed by a particle bound enzyme or enzyme system probably localized in the cell membrane (Davoren and Sutherland 1963) Other hormones and drugs such as vasopressin TSH LH ACTH glucagon and histamine are also able to stimulate the production of cyclic AMP The methylxanthines probably exert their action partly by inhibiting the breakdown of cyclic AMP (Fig 1 Cyclic AMP has also been found to influence a number of other enzymatic reactions and metabolic processes

The multifarious role which cyclic AMP appears to assume in hormonal and metabolic connection has led Sutherland and Robison (1966) to suggest that this compound takes a more general part in mediating the control by the cell membrane over some intracellular metabolic processes

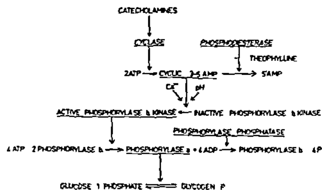


Fig 1 Schematic presentation of the mechanism by which the catecholamines activate phosphorylase *a* in skeletal and cardiac muscle according to Sutherland and Rall (1960) and Krebs *et al* 1959 ——— = Inhibition

Attempts at detailed studies of adenyl cyclase and the way in which it is activated by different drugs and hormones have met with difficulties since it is particle bound (Sutherland and Rall 1960) Catecholamines can stimulate the activity *in vitro* of adenyl cyclase containing particles (Murad *et al* 1962) Øye and Sutherland (1966) have presented evidence indicating that the catecholamine sensitive receptor site and enzymatic active site of adenyl cyclase may be dissociated Since several different hormones with varying structures are able to stimulate adenyl cyclase activity it is probable that either the cyclase system itself possesses several different receptors and/or that some of these drugs produce a common reaction which leads secondarily to adenyl cyclase activation Sutherland Robison and Butcher (1968) reported that insulin and prostataglandin reduced the cyclic AMP increasing action of the catecholamines in intact fat cells but not the action of catecholamines in a homogenate of fat tissue This finding may give some support to the assumption that adenyl cyclase activation in intact tissue may also be stimulated in a more indirect way

Tinder Boyne and Shoemaker (1964) demonstrated that the phopsphorylase activating effect of glucagon in the dog liver was preceded by an increased K efflux They suggested that a causative connection existed between increased K permeability and phosphorylase activation The relationship between the hyperglycemic and hyperkalemic effects of catecholamines and glucagon was studied by Ellis *et al* (1967) in the cat They concluded that the hyperkalemic effect was of the type of an adrenergic *α* receptor effect whereas the hyperglycemic effect was of the *β* type In the rat both adrenergic *α* and *β* receptor stimulation sems however to be involved in the hyperglycemic action of the catecholamines (Himns Hagen 1967) In the fed rat the hyperglycemic effect of the catecholamines seems to be an adrenergic *α* receptor effect (Fleming and Kenny 1964) and has been found to be only partly blocked by *β* receptor blocking drugs (Salvador April and Lemberger 1967 Ali Antonio and Haugaard (1964) reported that the phosphorylase activating effect of adrenaline in rat liver slices was blocked by both adrenergic *α* and *β* receptor blocking drugs In rat liver adrenaline may therefore activate phosphorylase both by *α* and *β* receptor mechanisms

Cahill *et al* (1957) observed that incubation of rat liver slices in a buffer solution with a high K^+ concentration reduced the phosphorylase activity in comparison with those incubated in normal buffer solution with Na^+

With reference to the findings outlined above it seemed of interest to ascertain whether variations in the ionic milieu influenced the total phosphorylase activity or the phosphorylase activating effect of the catecholamines In this paper experiments on the isolated diaphragm of the rat are reported

Methods

A rat was killed by a blow on the neck and its diaphragm excised The most ventral parts of the diaphragm were cut away It was then divided into four parts two lateral and two medial The two lateral parts formed a pair one being used for treatment with adrenaline and the other as a control The same procedure was followed for the medial parts The preparations were weighed and each was incubated in a test tube in 10 ml of the respective suspension solutions at 37° C The solutions were gassed with 5 % CO_2+95 % O_2 which gave a pH of 7 4

In the first series of experiments the one pair of diaphragm samples was incubated in ordinary Krebs Henseleits bicarbonate solution while the other pair was incubated in a bicarbonate buffer in which all Na ions had been replaced by K ions The compositions of the normal and high K^+ Krebs-Henseleits bicarbonate buffers are shown in Table I After 15—30 min preincubation adrenaline in a final concentration of $1 \cdot 10^{-5}$ g/ml was added to one of the preparations and 3 10 or 15 min later both the adrenaline and control preparations were analyzed for phosphorylase In some experiments adrenaline was replaced by $1 \cdot 10^{-5}$ L-isoprenaline In some tests the concentration of adenosine triphosphate (ATP) and of creatine phosphate (CrP) were analyzed in parts of diaphragm preincubated for 15 min in normal or high K^+ Krebs buffer solution Adrenaline (10^{-5} g/ml) was then added and after a further 15 min the control and adrenaline treated parts of the diaphragm were frozen in frigen 11 at −80° C and analyzed for ATP and CrP with enzymatic methods (Adam 1962 Beviz *et al* 1965)

In other series the phosphorylase activating effect of adrenaline was determined after 30—60 min preincubation in a medium in which the sodium chloride had been exchanged for choline chloride (Table I)

In experiments with Ca^{++} free Krebs-Henseleits bicarbonate buffer the effect of adrenaline on the phosphorylase activity was determined in one of the pair of preparations after 2 1/2—3 hrs preincubation in normal Krebs Henseleits bicarbonate buffer The other preparations were first incubated for 2 1/2—3 hrs at 37° C in Ca^{++} free buffer solution (Table I) before

TABLE I Composition of solutions used in this investigation Concentration in mM/l

Solution	NaCl	KCl	$CaCl_2$	$MgSO_4$	KH_2PO_4	NaH_2PO_4	$NaHCO_3$	$KHCO_3$	Glucose
Krebs Henseleit s bicarbonate buffer	120 0	4 7	2 5	1 2	—	1 2	25	—	5 6
K rich buffer	—	124 7	2 5	1 2	1 2	—	—	25	5 6
Ca free buffer	122 5	4 7	—	1 2	—	1 2	25	—	5 6
Na free choline buffer HCl to pH 7 35	— Choline chloride 120 0	4 7	2 5	1 2	1 2	—	— Choline bicarbonate 25	—	5 6

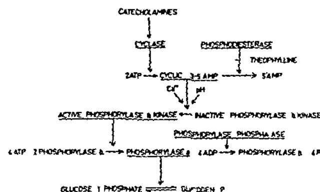


Fig 1 Schematic presentation of the mechanism by which the catecholamines activate phosphorylase *a* in skeletal and cardiac muscle according to Sutherland and Rall (1960) and Krebs *et al* 1959 — — — = Inhibition

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TABLE II. Influence of the composition of the buffer solution on the total phosphorylase activity and the phosphorylase *a* activity of isolated rat diaphragm. Parts of the diaphragm were incubated in Krebs-Henseleit's bicarbonate solution and other parts in K^+-rich, choline or Ca^{2+}-free buffer. The total phosphorylase activity was determined in the presence of 0.001 M AMP, the phosphorylase *a* activity in absence of AMP and expressed in per cent of the total activity. Significance of the effect is denoted by * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$

Experimental condition	number of tests	Krebs-Henseleit's solution: Total activity mgP/g min	Basal phosphorylase *a* activity per cent	Change of activity after incubation in altered buffer solution: Total activity	Phosphorylase *a* activity
K^+-rich buffer					
Incubation for 2 min	10	1.1 ± 0.04	15.8 ± 2.5	−0.14 ± 0.04**	−6.2 ± 2.2*
30 min	8	0.96 ± 0.03	13.5 ± 0.9	−0.07 ± 0.04	−3.3 ± 1.1*
Choline buffer					
Incubation for 30 min					
(high basal values)	7	1.51 ± 0.10	27.2 ± 4.9	−0.15 ± 0.06*	−13.1 ± 4.6*
30 min (low basal values)	10	0.87 ± 0.07	11.6 ± 1.5	−0.47 ± 0.08***	−1.2 ± 1.4
Ca^{2+}-free buffer					
Incubation for 180 min	5	0.34 ± 0.06	23.9 ± 1.1	−0.04 ± 0.07	−12.1 ± 3.4*

reduced (Table III). After 15 min incubation with adrenaline in high K^+ solution the effect of the adrenaline had completely disappeared. The progressively decreasing effect of adrenaline with increasing periods of incubation in high K^+ could have been due to the fact that the preincubation in K^+-rich solution was too short for a uniform ionic distribution to have had time to take place throughout the muscle. When the preincubation period was extended to 30 min the adrenaline did not induce any phosphorylase activation after 3 min of incubation (Table III). The phosphorylase activating effect of isoprenaline was also blocked in a high K^+ medium.

The influence of adrenaline on the concentration of ATP and CrP of diaphragm in normal and high K^+ media

The phosphorylase activating effect of the catecholamines is a reaction utilizing ATP (Fig. 1) and may be inhibited when high energy phosphates are lacking.

We therefore investigated the question of whether the ATP- and CrP-concentrations were affected in high K^+ solution. The results of these experiments are given in Table IV. The concentrations of high energy phosphate compounds were unchanged in high K^+ solution. In normal Krebs solution adrenaline induced an increase in the ATP and CrP concentrations in accordance with earlier results (Mohme-Lundholm, Svedmyr and Vamos, 1967). This increase was not observed in

TABLE III The influence of adrenaline (10^{-6} g/ml) and isoprenaline (10^{-6} g/ml) on the phosphorylase activity in parts of rat diaphragm preincubated in Krebs-Henseleits bicarbonate buffer solution in comparison to the effect on parts incubated in K^+-rich, choline or Ca^{++} free buffer solutions

Experimental condition	number of tests	Krebs-Henseleit's solution: Total activity mgP/g/min	Krebs-Henseleit's solution: Basal phosphorylase *a* activity per cent	Change of *a* activity after catecholamines in: Krebs solution	Change of *a* activity after catecholamines in: K^+-rich or choline or Ca^{++}-free buffer	Difference in catecholamine effect Krebs K^+-rich or choline or Ca^{++}-free buffer
K^+-rich buffer Preincubation 15 min,						
adrenaline 3 min	5	0.99±0.05	15.4±2.3	23.0±7.3*	7.0±3.0*	16.0±8.0
adrenaline 10 min	5	0.60±0.08	17.4±1.8	20.2±4.5*	4.6±1.1**	15.6±4.6**
adrenaline 15 min	5	0.68±0.08	17.0±1.7	13.4±3.3*	−1.0±2.4	14.4±4.1**
isoprenaline 15 min	4	0.68±0.06	16.7±2.3	15.7±2.3*	−3.3±1.2	17.7±1.7**
Preincubation 30 min,						
adrenaline 3 min	9	0.73±0.08	11.3±1.3	9.7±1.5**	−1.0±2.2	10.7±2.6***
Choline buffer Preincubation 30 min						
adrenaline 3 min	7	1.51±0.10	27.2±4.9	16.4±2.3***	2.7±0.8**	13.7±2.5***
Ca^{++} free buffer *Preincubation* 180 min						
adrenaline 15 min	5	0.34±0.06	23.8±1.1	27.5±5.7**	21.4±6.0*	6.2±8.2

high K buffer. It seemed probable that the increase in the high energy phosphate compounds of the diaphragm was connected with the phosphorylase activating and glycogenolytic effect of adrenaline.

Influence of adrenaline on the phosphorylase activity in choline buffer medium

The reduction of the basal phosphorylase *a* activity and the phosphorylase activating effect of catecholamines might be attributed either to the presence of high K or absence of Na. To ascertain which of these alternatives was valid we exchanged sodium chloride in Krebs- Henseleits solution for choline chloride (Table I). After preincubation of the diaphragm for 30—60 min in the sodium-free solution the total phosphorylase activity fell significantly (Table II). There was a significant reduction of the phosphorylase *a* activity when the basal value was high but not when it

TABLE IV Influence of the composition of the buffer solution and adrenaline (10^{-6} g/ml) on the ATP and CrP content (in µmoles/g wet weight) of parts of isolated rat diaphragm The diaphragm was preincubated for 30 min in the different solutions time of incubation with adrenaline 15 min

Experimental condition	Control values		Change after adrenaline	
	ATP	CrP	ATP	CrP
1 Krebs-Henseleit's buffer solution (n = 8)	0 77 ± 0 08	1 44 ± 0 35	0 67 ± 0 16***	1 40 ± 0 54*
2 K^+-rich solution (n = 8)	0 91 ± 0 15	1 83 ± 0 45	−0 09 ± 0 23	−0 60 ± 0 61
Difference 1—2	−0 14 ± 0 19	−0 39 ± 0 42	0 76 ± 0 30*	2 00 ± 0 81*
3 Krebs Henseleit's buffer solution (n = 14)	0 47 ± 0 09	0 72 ± 0 16	—	—
4 Choline buffer solution (n = 14)	0 32 ± 0 06	0 47 ± 0 08		
Difference 3—4	0 14 ± 0 10	0 24 ± 0 16	—	—

was low (Table II) The effect of adrenaline was also significantly reduced although not totally blocked in choline medium (Table III)

After incubation for 30 min in choline buffer there was some reduction of the high energy phosphate compounds in the diaphragm in comparison with muscle pieces incubated in Krebs-Henseleits buffer solution for the same period of time (Table IV), but the effect was not statistically significant This action may be a complicating factor as it can influence the synthesis of phosphorylase *a* (Fig 1) and thereby secondarily change the action of adrenaline

Influence of Ca -absence on the phosphorylase a activating effect of adrenaline

Ca -ions can activate phosphorylase *b* kinase (Krebs Grant and Fischer 1959) (Fig 1 and thereby influence the phosphorylase *a* activity To study the possible importance of Ca ions for the phosphorylase activating effect of adrenaline a series of experiments were performed in which diaphragm was incubated for 180 min in ordinary or Ca -free Krebs solution at 37° C before addition of adrenaline The basal phosphorylase *a* activity fell in the preparations incubated in Ca free solution but the phosphorylase *a* activating effect of the adrenaline was not altered with certainty (Table II and III)

Influence of cyclic AMP on the phosphorylase a activity in Krebs Henseleits buffer solution and after replacing Na by K or choline

The blockade of the phosphorylase *a* activating effect of adrenaline in K -rich solution could be attributed either to an inhibition of the stimulating action of adrenaline on the cyclic AMP production or to blockade of the effect of cyclic AMP on the phosphorylase activating process in the presence of K or absence of Na^+ In

TABLE V. Influence of cyclic AMP (CA, 1 or 5 µmoles/ml) on the phosphorylase activity in parts of isolated rat diaphragm incubated in Krebs-Henseleit's, K^+ rich or choline buffer solution.

Incubation *solution*	Total phosphorylase *activity mgP/g/min*		Phosphorylase *a* activity *per cent*	
	Control	Change after CA	Control	Change after CA
1 µmole CA/ml				
1 Krebs-Henseleit's buffer solution (n = 19)	1.23±0.04	0.03±0.06	31.1±1.8	4.0±1.0**
2 K^+ rich buffer solution (n = 19)	1.04±0.09	−0.03±0.12	21.2±2.1	−0.4±1.5
Difference 1—2	—	—	—	4.4±1.8**
5 µmole CA/ml				
3 Krebs-Henseleit's buffer solution (n = 10)	0.87±0.07	−0.03±0.04	11.6±1.5	+8.8±2.9*
4 Choline buffer solution (n = 10)	0.45±0.04	−0.03±0.04	10.4±1.80	+2.2±1.0
Difference 3—4	0.42±0.08***	0±0.05	1.2±1.4	6.6±1.6***

In testing the first alternative we found that adrenaline still increased the cyclic AMP content of rat diaphragm incubated in high K^+ medium (Lundholm, Rall and Vamos 1967). To test the latter alternative we preincubated four parts of diaphragm in normal solution for 30 min and then added cyclic AMP as sodium salt in a final concentration of 1 µmole/ml to two of the parts. After 15 min there was a rather small but significant increase in the phosphorylase *a* activity (Table V). When the muscle was preincubated in K^+ rich solution for 30 min cyclic AMP had no effect on the phosphorylase *a* activity. The difference between the effects of cyclic AMP in normal and high K^+ solutions was significant (Table V).

In another series of tests parts of the diaphragm were preincubated for 30 min in normal buffer solution or in a buffer medium where Na^+ was replaced by choline. Cyclic AMP in a concentration of 5 µmoles/ml increased the phosphorylase *a* activity more than 1 µmoles/g (Table V). In choline buffer the effect of cyclic AMP was significantly less than in normal buffer solution.

Discussion

The reported experiments indicated that variations in the ionic milieu influenced the phosphorylase activity of the isolated rat diaphragm in different ways. Substitution of Na^+ in Krebs-Henseleit's bicarbonate buffer for K^+ reduced the basal phosphorylase *a* activity and inhibited the phosphorylase *a* activating effect of the catecholamines but did not influence the total phosphorylase activity. There was no

change of the concentration of the high energy phosphate compounds Substitution of choline for Na resulted in the first two effects but also reduced the total phosphorylase activity, there was eventually some reduction of the high energy phosphate compounds In Ca^{++} free solution the basal phosphorylase *a* activity was reduced but the total phosphorylase activity and the effect of the catecholamines was unchanged It is improbable that all these effects can be explained by the presence or lack of one single ion or by blockade of the action of cyclic AMP In the tests with choline the reduction of the high energy compounds may be of some importance for the observed effects Posner Stern and Krebs (1964) showed that the phosphorylase *a* activation following electrical stimulation of skeletal muscle of the rat was not accompanied by an increase in the level of cyclic AMP Adrenergic β receptor blocking drugs which inhibit the catecholamine induced stimulation of cyclic AMP formation (Sutherland and Robison 1966) did not reduce the basal phosphorylase *a* activity of isolated rat diaphragm (Mohme Lunholm and Svedmyr 1964 Ali *et al* 1964)

The phosphorylase *a* activity of skeletal muscle is therefore regulated by other factors besides cyclic AMP Our observations that the presence of high K or the absence of Ca^{++} in the buffer solution reduced the basal phosphorylase *a* activity especially when it was high may indicate that changes of ionic distribution over the cell membrane may be of importance for the regulation of the phosphorylase *a* activity The presence of sodium seemed also to be needed for the phosphorylase *a* activating action of cyclic AMP The phosphorylase *a* activating effect of both catecholamines and cyclic AMP was reduced or blocked when Na in the buffer solution was replaced by K or choline In K rich solution adrenaline still increased cyclic AMP formation which was also increased by K itself (Lundholm *et al* 1967) It is therefore probable that high K or lack of Na blocked the action of cyclic AMP on the phosphorylase *a* activating process The location of this blockade is unknown It is not probable that a blockade of the transport function of the cell membrane was involved as adrenaline still increased the intracellular concentration of cyclic AMP in high K solution As the reaction leading to activating of phosphorylase *b* kinase is influenced by other ions such as Ca and H (Fig 1 Krebs Graves and Fischer 1959) an effect on this reaction may be considered It also seems probable that the reduction of the basal phosphorylase *a* activity in Ca free solution may be located to this reaction

The blockade of the phosphorylase activating effect of the catecholamines in rat diaphragm suspended in high K media seems to be a rather specific effect In isolated rat liver slices (Cahill *et al* 1957 or intestinal smooth muscle of the rabbit (Andersson and Mohme Lundholm 1968) the catecholamines still stimulated the phosphorylase activity in high K solution

There is evidence that different drugs may also inhibit the *action* of cyclic AMP on metabolic processes Northrop and Park (1964) reported that DHE inhibited the hyperglycemic action of injected cyclic AMP in the rat High concentrations of both adrenergic α and β receptor blo[illegible]

the lipolytic action of dibutyryl AMP in epididymal adipose tissue of the rat (Aulich Stock and Westerman 1967) DHE and adrenergic β-receptor blocking drugs in lower concentrations are also reported to block the stimulating effect of adrenaline on adenyl cyclase (Sutherland and Robison 1966) Both DHE and DCI had however stimulating actions of their own on adenyl cyclase (Murad *et al* 1962) Depending on the dose and the kind of adrenergic β-receptor blocking agent, these drugs may have the following actions 1 stimulation of adenyl cyclase 2 inhibition of the stimulating action of catecholamines and other hormones on adenyl cyclase 3 inhibition of the action of cyclic AMP 4 They may possess a local anesthetic and cardiodepressive effect of their own independent of adrenergic β-receptor blocking properties (Levy 1968) An interesting observation demonstrating the complex action of DCI was reported by Ali *et al* (1964) who observed that in a low concentration it stimulated the phosphorylase *a* activity of isolated rat diaphragm whereas in a higher concentration it blocked its own effect Nicotinic acid seems to have a more specific blocking action on some effects of cyclic AMP In tests on brown adipose tissue we found that nicotinic acid blocked the lipolytic action of cyclic AMP and noradrenaline but not the calorigenic or lactate increasing actions of these drugs (Bevtz and Mohme Lundholm 1967) Cyclic AMP may thus influence some kind of intracellular "cyclic AMP receptor mechanism or mechanisms which can be blocked by drugs and influenced by the ionic composition of the buffer solution.

As mentioned previously K ions increased the cyclic AMP level in the rat diaphragm (Lundholm *et al* 1967) but there was no activation of phosphorylase *a* as K (or lack of Na) inhibited at the same time the phosphorylase *a* activating action of cyclic AMP K ions therefore probably had both an activating and inhibitory action on the processes leading to phosphorylase *a* activation and resembled the action of DCI It was of interest to study the question of whether the stimulating and inhibiting actions of K had the same time constant. As can be seen (Table II) the phosphorylase *a* activity had fallen both after 2 and 30 min of incubation in K rich buffer indicating that the blocking action started very rapidly The total phosphorylase activity had increased after 2 min, however

Danforth and Helmreich 1964 observed however that the phosphorylase *a* activity of frog skeletal muscle increased proportionally to an increase in the extra cellular K concentration They thought that Ca liberated from the cell membrane was responsible for the phosphorylase *a* activation. The possibility that some effect of K might be attributed to an increased formation of cyclic AMP may however be considered. In isolated brown fat tissue K stimulated the oxygen consumption and release of FFA and glycerol effects which were also produced by catecholamines and addition of cyclic AMP (Bevtz *et al* 1968

As is evident from this discussion there are similarities between the action of catecholamines and adrenergic blocking drugs and the action of ions on the formation and activity of cyclic AMP It is an interesting question whether these similarities are only of a superficial nature or where a causal relationship exists between the action of drugs and ions

Clausen (1966) studied the relationship between cations and carbohydrate metabolism in the rat diaphragm He found that when Na^+ in the buffer was replaced by K^+ or choline both the glucose uptake lactate production and incorporation of glucose into glycogen were diminished Absence of Na^+ therefore seems to influence both the phosphorylase activity and glucose metabolism of rat diaphragm The possible relationship betwen these effects requires further study, however

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The Effects of L-Triiodothyronine on the Self-Selected Circadian Rhythm of Rest and Activity in the Canary

By

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Abstract

WAHLSTRÖM, G *The effects of L-triiodothyronine on the self-selected circadian rhythm of rest and activity in the canary* Acta physiol scand 1969 75 199—212

Canaries were used as experimental animals The birds were kept singly Each cage was [illegible] used one perch in the [illegible] circadian rhythm of [illegible] the starting point of [illegible] ose at different times during the activity Oral doses up to 10 mg/kg given early in the activity did not influence the length of the circadian period There was an uncertain increase in activity Similar doses given late in the activity increased the length of the circadian period The duration of activity was increased and the rest was decreased The changes were maximal in the period after the one in which T3 was given and gradually disappeared during approximately a week The [illegible] of circadian period and activity was independent of the pre-experimental value Thyrotropic hormone (1—2 IU) in a few birds caused a small increase in the duration of the circadian period in which it was given

Many living organisms are adapted to and governed by the external changes between light and darkness The processes behind the adaptation are not merely passive ones as organisms show a rhythm of approximately 24 hours (circadian rhythm) in constant environments with regard to light and temperature (for examples see Aschoff 1963) The active part of the adaptation process (the clock mechanism) must derive its energy from the metabolism of the organism studied It is in this connection interesting to know in which manner manipulations of the thyroid hormones influence the clock mechanism in vertebrates The data which have appeared in the literature so far are surprisingly scarce (for discussion of the relevant literature see Bunning 1963 chap 8 Roberts 1965 and Rensing 1965) Most of the work has been performed in insects with contradictory results

In the present paper the effects of L-triiodothyronine on the selfselected circadian rhythm of light and darkness have been studied in the canary Some examples of the

effects have been given earlier (Wahlström 1965a) and a preliminary note on the results has also been published (Wahlström 1967). The results definitely show that it is possible to influence the circadian rhythm in the canary by L-triiodothyronine. The duration of activity and of the circadian period were increased. Opposite results obtained by administration of ^{131}I will be presented in another paper (Wahlström 1969).

Methods

The method has been described in detail elsewhere (Wahlström 1964). The canaries (*Serinus canarius*) were kept singly in wooden cages. Each cage was illuminated by a common electrical bulb (220 V, 75 W) and in the compartment housing the bird there were two perches. One perch was connected to the lamp in such a manner that the light was extinguished when the bird used the perch. The bird could thus choose between light and darkness by jumping from or onto this perch. The other perch was used only to record the locomotor activity of the bird while the light was on. Male canaries were obtained from local dealers and trained. All birds used had participated in other experiments and were well adapted to their cages. Water and food (commercial canary seed mixture) was available at all times.

The waking up times has been used as the starting point to calculate the circadian period which thus usually consists of one activity period and one rest period. Sometimes short rest periods (only those larger than 0.5 hours have been counted) were interspaced in the main activity. These rest periods has been added to the main rest period ("gross activity and rest" Wahlström 1964).

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In the oral experiments L-triiodothyronine (T3) was administered through a stomach tube. In the circadian periods before and after the hormone administration distilled water was given in the same manner. This placebo treatment could not always be performed but in birds accustomed to the treatment these placebo administrations have no effect on the circadian rhythm (Wahlström 1964).

In the parenteral experiments T3 was either injected into the breast muscle or subcutaneously on the back between the wings. No placebo injections were performed in the present experiments but such injections have no influence on the self-selected rhythm (Wahlström unpublished).

Sodium L-triiodothyronine used in the oral and intramuscular experiments was obtained from Nyegaard & Co A/S Oslo. The concentration of the solution was 0.2 mg/ml. In the subcutaneous experiments sodium L-triiodothyronine obtained from Glaxo Lab. Ltd. was used and it was diluted prior to use to a concentration of 0.2 mg/ml.

Thyrotropic hormone (Actyron®) was administered subcutaneously in a few experiments. It was obtained from Ferring AB and diluted in such a manner that 40 µl contained 1 IU of thyrotropine. The solvent prepared by Ferring AB was used (Phenol 15 mg, glycose q.s. and aqua sterilisata ad 5 ml). Actyron® is prepared from sheep and has according to Ferring only thyrotropic properties. It is not chemically pure.

In all experiments a pre-experimental period of five circadian periods was first recorded. T3 (or thyrotropine) was given as a single dose during the activity in circadian period no. 0. Behavioural changes were estimated by direct observation at irregular intervals after the hormone administration. For each experiment the changes in circadian rhythm induced were [illegible] circadian [illegible] their rest [illegible]

Results

A. *Experimental material and body weight changes*

L-triiodothyronine (T3) was administered orally in most of the experiments. The first experiments were performed with doses around 0.01 mg/kg. The doses were

TABLE I Number of experiments performed with different doses of L triiodothyronine at different times in the circadian period (number of birds in parenthesis)

Dose (mg/kg) and route of administration	Evaluated								Excluded
	1(i m)	1(s c)	1—5 (p o)	6(p o)	7(p o)	8(p o)	10(p o)	> 10 (p o)	
AM series	—	—	2(1)	5(3)	—	2(1)	—	—	5*
PM series	2(1)	7(7)	5(2)	19(2)	2(1)	12(5)	4(1)	2(1)	17**

AM series = T3 administered early in the activity PM series = T3 administered late in the activity *i m* = *intramuscular* *s c* = *subcutaneously* *p o* — *orally*

* 2 expts were excluded as the time interval was less than 2 weeks to the prior experiment

** 11 expts were excluded as the time interval was less than 2 weeks to the prior experiment

then increased in approximate steps of 2× until changes were seen in the circadian rhythm Usually two sometimes more experiments were performed on each dose level Experiments with doses of T3 less than 1 mg/kg showed no detectable change No birds died even after the largest doses

In Table I are given the number of experiments in which the administered doses were 1 mg/kg or more As the changes in the self selected circadian rhythm induced by T3 lasted approximately a week all experiments where the previous dose in the same bird was given less than a fortnight earlier have been excluded The total number of experiments excluded for this reason were 11 in the PM series and 2 in the AM series Three other PM experiments were excluded because the exact amount of drug given in these experiments was unknown Marked irregularities in the pre experimental periods and technical difficulties mainly with the recording made the remaining excluded experiments impossible to evaluate

A large number of experiments in the PM series with 6 and 8 mg/kg were per formed on one bird to study whether the spontanous changes which mainly occur in activity, rest and body weight over approximately a year (Wahlstrom 1964) had any influence on the response to T3 These experiments (n=23) were if possible performed every second week over approximately one and a half year

There was no large decrease in bodyweight after T3 In the birds which got 8 mg/kg of T3 orally the average decrease was 0 25 ± 0 23 g (S E) The number of experiments with adequate data were 12 The pre experimental value was obtained two days prior to the T3 administration and the post experimental value five days after the administration

B *Changes in behaviour*

There was no consistent change in behaviour after T3 admi[illegible] ex- periments with 8 mg/kg given orally in the PM series showed the [illegible]nges in the self selected circadian rhythm (see results part C) These [illegible]have

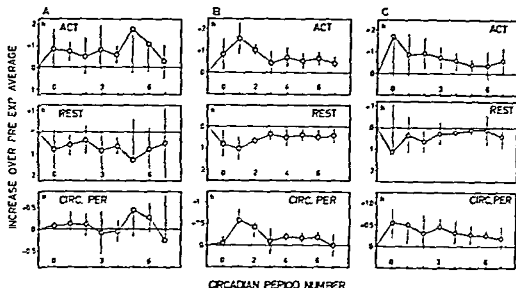


Fig 1 Effects of a single dose of L-triiodothyronine on the circadian rhythm. The dose was in A 6—10 mg/kg given orally early during the activity (AM) in B 6—10 mg/kg given late in the activity (PM) and in C 1 mg/kg given parentally late during the activity (PM). The number of experiments were in A 7 up to circadian period no 5 and then 6 in B 37 up to circadian period no. 4 36 in period no 5 34 in period no 6 and then 32 and in C 9 up to circadian period no 4 8 in period no 5 and 6 and then 7 Increases have been calculated from the averages of the 5 circadian periods prior to the hormone administration in period no 0 Vertical bars indicate twice the S E. The hormone was given on an average 5 59 h (A 9 27 h (B and 9 21 h (C) after the waking up in circadian period no 9 The mean pre-experimental (Pre-exp average for all experiments was activity (Act.) 11 72 h A 12 51 h B 11 09 h C rest 12 57 h (A 10.87 h B 12 13 h C) and circadian period Circ Per) 24 29 h A 23.38 h B 23 22 h C

been closely evaluated with regard to changes in locomotor activity on the day or night perch after the drug administration In 3 experiments out of 12 there was an increase in locomotor activity already during the circadian period in which the hormone was given (period no 0) In 5 out of 12 expts there was an increase in locomotor activity in the later parts of the activity period which followed the one in which T3 was administered No consistent increase in locomotor activity indicating an increased restlessness in the birds could thus be established after administration of T3 in doses which had effects on the circadian rhythm

C *Changes in the self selected circadian rhythm*

Only 2 expts were performed with 1—5 mg/kg of T3 given orally as an AM-dose These experiments showed no convincing effects of T3 on the circadian rhythm. Results of experiments with oral doses in the range 6—10 mg kg are shown in Fig 1A. There was no change in the circadian period and only an uncertain (but persistent) increase in activity and a corresponding decrease in rest T3 given early in the activity in doses up to 10 mg/kg thus did not show any convincing effects on the self-selected circadian rhythm

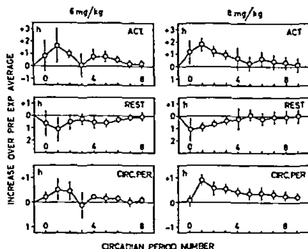


Fig ? Comparison between the effects of 6 and 8 mg/kg of oral L-triiodothyronine given late in the activity (PM) on the circadian rhythm. The number of experiments was in the 6 mg/kg series 19 up to circadian period no. 4 18 in circadian period no 5 17 in circadian period no 6 16 in circadian period no 7 and then 15 The number of experiments was in the 8 mg/kg series 12 up to circadian period no 6 11 in circadian period no 7 and 8 and then 9 Increases have been calculated from the averages of the 5 circadian periods prior to the hormone administration in circadian period no 0 Vertical bars indicate twice the S E. The hormone was on an [illegible] 0 in the 6 mg/kg series The [illegible] n pre-experimental (Pre Exp) [illegible] (Act) 17 78 h. rest 10 66 h [illegible] g series activity 12 29 h rest

T3 in doses between 1—5 mg/kg given orally late in the activity (PM-doses) induced some changes in the circadian rhythm The activity was increased in the period in which the drug was given and in the two that followed This increase was between 1 an 2 hrs There was a corresponding decrease in rest which was of the same magnitude In a few experiments this decrease was slightly smaller than the increase in activity There was thus only a small and uncertain increase in the circadian period (Data not shown)

In one case when 6 mg/kg was given as a PM dose the expected rest period did not appear Thus the bird lost the first night s sleep after drug administration In another case the bird lost the second night s sleep As the time from the waking up prior to this long activity period to the next waking up was approximately twice the normal circadian period (55 70 and 49 78 hrs) these experiments were included The circadian period was taken as the time between the two waking up times divided by two and the first circadian period of these two was at the same time counted as activity as the rest period belonging to it had disappeared As the number of experiments was large these 2 expts have very little influence on the average in the 6—10 mg/kg PM series

Fig 1B shows that 6—10 mg/kg T3 orally given as PM-doses clearly influenced the circadian rhythm There was an increase in activity which was largest in the period following the drug administration and then gradually disappeared A slight increase was still evident a week after the hormone administration The rest period showed smaller changes in the opposite direction The circadian period was increased and this increase was also largest in the period following the hormone ad

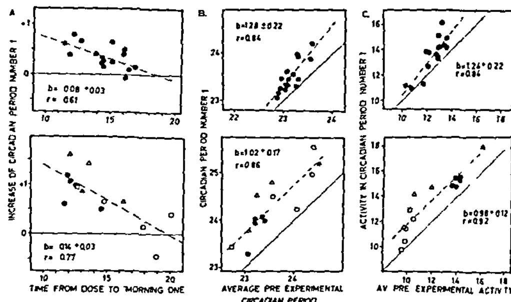


Fig 3 The relationship between time from dose to 'morning one' (the waking up which starts circadian period no 1) and L triiodothyronine induced increase in the length of circadian period no 1 (A) and between pre experimental values and values in circadian period no 1 (B and C) The duration of circadian period no 1 in (B) was recalculated with the regression found in A to obtain a constant time (14 42 h) from dose to 'morning one' in all experiments In the upper part the material consists of experiments where 6 mg/kg L-triiodothyronine was given to the same bird All experiments (n=16) belonged to the M series In the lower part the material consists of experiments where 8 mg/kg of L triiodothyronine was given to different birds Twelve out of 14 experiments belonged to the PM series The hormone was given in circadian period no 0 The experiments performed in the same individual are indicated with similar symbols The dashed line indicates the regression line b=linear regression coefficient r=correlation coefficient Time scales in hours

ministration The duration of the change is uncertain but it was not more than a week The slight increase in period no 4—6 could be real as a similar more marked increase was obtained in hypothyreotic birds (Wahlstrom 1969)

In Fig 2 the results of the PM experiments with 6 and 8 mg/kg of T3 are given separately The increase in the circadian period was larger and had a longer duration in the 8 mg/kg experiments As there was no change in circadian period in the experiments with doses below 5 mg/kg the change in the circadian period seems to be dose dependent in the dose interval studied No corresponding dose dependence could be established with regard to changes in activity and rest in this dose interval However, the 2 expts with long activity periods belonged to the 6 mg/kg series which makes the activity figures uncertain in the first three periods (number 0—2) in this series

To get a rough estimate of the losses accompanying oral administrations one series with parenteral administration was performed T3 was on 7 occasions given s.c. and on 2 occasions given i.m. As there was no substantial difference between these two manners of administration the data has been pooled The hormone (1

mg/kg) was administered late in the activity (PM) The results are given in Fig 1C The effects obtained were similar (except in circadian period number 0) to those obtained after oral administration of 6—8 mg/kg (Fig 1B) Approximately 10—20 per cent of an oral dose thus seems to be utilized

When T3 was given late in the activity of circadian period no 0 circadian period no 1 was clearly increased (Fig 1B) No similar change was seen when T3 was given early in the activity (Fig 1A) This indicate that the time in the circadian period at which the hormone was administered influenced the induced change As the maximal increase seemed to occur in period no 1 the time between the hormone administration and the waking up which starts period no 1 morning one (see Fig 4) was choosen as the abscissa on which the changes in circadian period no 1 were plotted as ordinates In Fig 3A this plot is shown for two sets of experiments In Fig 3A upper part experiments with 6 mg/kg of T3 given to the same bird throughout have been used All experiments belonged to the PM series In Fig 3A lower part all experiments with 8 mg/kg of T3 have been used 12 out of 14 experiments belonged to the PM series

Fig 3A clearly shows that in both sets of experiments the change induced in the circadian period depended on the time of drug administration Both linear regression coefficients were significantly different from zero There was however no significant difference between the two coefficients indicating that the pooling of several birds in Fig 3A lower part did not cause much distortion

If the clock regulating the self selected circadian rhythm runs continuously and furnishes the waking up impulse with a biological variation then a variability in morning one could cause a regression similar to that seen in Fig 3A This is schematically shown in Fig 4A The variability (d) in case 1 is added to circadian period no 1 and at the same time subtracted from the time between dose and morning one In case 2 the opposite happens Thus a spurious regression results The alternative hypothesis that the clock is restarted at every waking up and then runs for a specified time (subject to biological variation) would not induce the kind of regression found in Fig 3A

Whether the regression in Fig 3A is due to the clock behaving according to the first hypothesis can be tested by calculating the regression between time from dose to morning one X and the increase in circadian period no 0 y If the first clock hypothesis is true d in Fig 4A will be subtracted from (case 1) or added to (case 2) both x and y causing a positive regression of the same magnitude as the one shown in Fig 3A The linear regression coefficients were however $-0\,074 \pm 0\,032$ for the data in Fig 3A upper part and $-0\,009 \pm 0\,038$ for the data in Fig 3A lower part It is thus unlikely that the regression seen in Fig 3A could be explained by the first clock hypothesis

The time from the dose to morning one includes a variable which could be responsible for the correlation obtained in Fig 3A T3 was usually given approximately 2 hours prior to the expected roosting in the PM series The expected time from roosting is judged from the preexperimental behaviour This means (see Fig

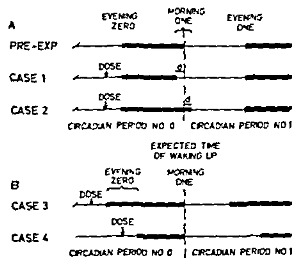


Fig 4

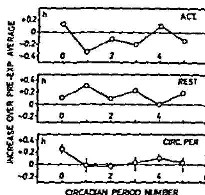


Fig 5

Fig 4 Schematical representation of variables which could influence the regression found between time from dose to "morning one" and increase in circadian period no 1 (Fig 3 A) For further explanation see text

Fig 5 Effects of a single subcutaneous dose of thyrotropic hormone (1—2 IU/bird) given late during the activity (PM) on the circadian rhythm. The number of experiments was 3 (2 with 1 IU) Increases have been calculated from the averages of the 5 circadian periods prior to the hormone administration in circadian period no 0 Vertical bars indicate the range The range of activity and rest were larger than the permissible space The hormone was given on an average 8 06 h after the waking up in circadian period no 0 The mean pre-experimental (Pre Exp) average for all experiments was Activity (Act) 12.58 h rest 11 13 h and circadian period (Circ Per) 23 71 h

4B) that experiments with a long time between the dose and "morning one" (case 3 Fig 4B) tend to consist of experiments with long pre-experimental rest periods ($r = 0.61$ DF = 14 for the data in Fig 3A upper part and $r = 0.77$ DF = 12 for the data in Fig 3A lower part) If the pre-experimental rest is kept constant by calculating partial correlation coefficients $r_{1\cdot 3}$ between the variables in Fig 3A upper part was 0 42 ($P > 0.05$ DF = 13) and in Fig 3A lower part it was 0 74 ($P < 0.01$ DF = 11) The corresponding partial correlations ($r_{32.1}$) between pre-experimental rest and changes in circadian period no 1 (the time from dose to "morning one" kept constant) were distinctly less −0 27 ($P > 0.05$ DF = 13) in Fig 3A upper part and 0 34 ($P > 0.05$ DF = 11) in Fig 3A lower part The main but possibly not the only factor related to the response in circadian period no 1 thus seems to be the time from the dose to "morning one"

The values of the regression coefficients in Fig 3A have been used to recalculate the observed changes in length of circadian period no 1 so as to eliminate the influence of the fact that T3 was administered at different times prior to the waking up at "morning one" Thus a standard time interval between dose and "morning one" was established mathematically Fig 3B shows that after correction in this manner circadian period no 1 was increased by T3 with approximately the same amount irrespective of the pre-experimental value of the circadian period Since there is no

TABLE II Correlations between pre experimental weight (X) and some variables (Y) in the circadian rhythm prior to and after L-triiodothyronine (T3) administration

Y	Data from one bird DF = 13* 6 mg/kg		Data from different birds DF = 12, 8 mg/kg	
	r	P	r	P
Pre-experimental activity	—0 73	*<0 01*	*+0 66*	*<0 01*
T3 induced increase in activity (period no 0)	—0 04	>0 05	—0 36	>0 05
T3 induced increase in activity (period no 1)	—0 33	>0 05	—0 32	>0 05
Pre experimental circadian period	—0 15	>0 05	—0 30	>0 05
T3 induced increase in circadian period no 0	—0 03	>0 05	0 36	>0 05
T3 induced increase in circadian period no 1	—0 15	>0 05	0 07	>0 05

DF=degrees of freedom
P =from Snedecor 1956 p 174
r =correlation coefficient
* 1 expt excluded as no pre experimental weight was recorded

reason to suspect that the amount of T3 added to the endogenously produced thyroid hormones depends on the length of the pre experimental circadian period the results in Fig 3B indicate that the sensitivity to T3 administrations of the clock mechanism was independent of the pre experimental value within the range tested With the doses of T3 used there was thus no evidence for the existence of a maximal length of the circadian period *corresponding to the minimal length* obtained in ^{131}I experiments (Wahlstrom 1969) However the largest period seen after T3 are still not longer than those which (rarely) occur in untreated animals

The change in activity induced (in periods nos 0 and 1) by T3 were also analyzed with regard to influence of the time of the dose administration The *experiments* in Fig 3 were used There was no significant correlation between change in duration of activity and any of the variables (1) time from dose to morning one (2) time from dose to expected roosting in circadian period no 0 and (3) time from dose to actual roosting in circadian period no 0 *evening zero As just discussed there* was such a correlation between variable *(1) and the changes in* circadian period (Fig 3A) There is thus a difference between duration of activity and circadian period *in this respect*

Fig 3C shows that the increase in duration of activity induced by T3 in circadian period no 1 was roughly independent of the duration of the pre-experimental activity *(the b-values do not differ significantly* from 1 0) The results were similar to those obtained with the circadian period (Fig 3B) The correlation between changes in activity and recalculated changes in circadian *period (both in period*

no 1) was 0 72 ($P<0\,01$, DF=28) for all experiments shown in Fig 3 pooled This is not surprising since the activity period is part of the circadian period

Since T3 influenced the circadian rhythm and thyroid hormones are known to influence the weight of small birds (see discussion) the correlation coefficients between pre-experimental weight and different variables in the circadian rhythm prior to and after T3 are of interest. These correlation coefficients are given in Table II Data for one bird were obtained from the same material as that in Fig 3 upper part (no weight data in one experiment), and the data for "different birds from the same material as that in Fig 3 lower part. Only the correlations with pre-experimental activity were significant but with different signs in the two materials

C *Effects of thyrotropine*

Five birds have been given thyrotropine 1—2 IU/bird Two of these birds died within a few hours of the administration In the other experiments the birds were very subdued for about 2 hours after the hormone administration There was also less jumping activity after it.

The results of the experiments which could be evaluated are given in Fig 5 Due to the small number of experiments no definite conclusions regarding the effects can be reached Nevertheless these experiments indicate that stimulation of the thyroid in this manner caused an increase in the first circadian period The changes in activity and rest were very small and the variability large

Discussion

In mammals there seems to be a difference in relative activity between L-triiodothyronine (T3) and L-thyroxine Measured by various means T3 is 2—10 times more active (Selenkow and Asper 1955 Barker 1955 Money Kumaoka and Rawson 1960 There is also a longer latent period after L-thyroxine administration (Pitt Rivers and Tata 1959 p 90 The actions of T3 and L-thyroxine show no such differences in birds (Shellabarger 1955 Newcomer 1957 The possible cause of this difference between mammals and birds could be the lack of a thyroxine binding globulin in birds (Tata and Shellabarger 1959 Shellabarger and Tata 1961 This explanation has been challenged by Dubowitz, Myant and Osirio (1962) after in vitro experiments However concomitant administration of the thyroxine binding globulin and L-thyroxine in vivo decreased the fast component of the disappearance curve of L-thyroxine in the chicken Shellabarger and Tata 1961 No corresponding change was obtained with T3 There are thus indications that in the chicken L-thyroxine and T3 may be equally active If results from the chicken can be applied to other birds in a study of effects of thyroid hormones it is not as critical as in mammals which one is choosen.

To obtain measurable responses in an intact animal doses exceeding the normal secretion of thyroid hormones must presumably be given since the secretion is in

hibited by the exogenous hormone (for references see Soderberg 1959) In the present experiments the doses were increased until certain effects on the self selected circadian period were obtained These doses did not cause any dramatic change in locomotor activity nor any overt behavioural signs There was furthermore no large decrease in bodyweight even in the birds treated with 8 mg/kg T3 orally (results part A) The post experiment weight measurements were maybe performed too late after the administration the maximal decrease in weight in small birds after thyroid hormone seem to occur on the third day after administration (Schildmacher and Rautenberg 1952, Wagner and Muller 1963) The administered doses of T3 thus had little general effect on the birds and a comparison with the level of secretion in small birds is warranted

No direct data on the daily secretion of thyroid hormones in canaries have been found in the literature Several effects of thyroid hormones have nevertheless been studied in small birds The moulting process is influenced by thyroid hormones in birds (Hohn 1950, Hohn 1961 King and Farner 1961) The manner in which the hormones affect the process is however, not fully understood (Hohn 1961) The liminal parenteral dose of presumably DL-thyroxine needed to obtain changes similar to the moult in birds not ready to moult has been found to be 1 mg/bird in the canary (Takewaki and Mori 1944), 0 15 mg/bird in the English sparrow *(Passer Domesticus)* (Miller 1939), 0 2 mg/bird in the brambling *(Fringilla Montifringilla)* (Wagner and Muller 1963) and between 0 2 and 0 4 mg/bird in the chaffinch *(Fringilla Coelebs)* and in one brambling (Schildmacher and Rautenberg 1952) These doses are high compared with a recalculated parenteral dose of approximately 0 04 mg/bird of DL-triiodothyronine used in the present experiments (1 mg/kg of T3 to a bird with an assumed b w of 20 g) A priming of the organism by previous action of sex hormones may however be an essential factor in the normal moulting process (Hohn 1961) If this is the case then the doses needed to obtain similar changes in birds not ready to moult could be higher than the physiological range

Increase in oxygen consumption and decrease in bodyweight are two other effects which have been studied after thyroxine administration in small birds. In male greenfiches *(Chloris Chloris)* 0 08 mg/bird had no effect but 0 2 mg/bird in male chaffinches *(Fringilla Coelebs)* seemed to be the minimal effective dose of parenteral thyroxine (Schildmacher and Rautenberg 1952) The bodyweight was influenced in the brambling by 0 1 mg/bird (Wagner and Muller 1963) and 0 15 mg/bird of thyroxine in the English sparrow *(Passer Domesticus)* increased the oxygen consumption (night value) by 24 per cent (Miller 1939) These doses are only slightly larger than those used in the present experiments It is thus probable that the doses used here are not enormously much larger than the physiological range This is further indicated by the thyrotropine experiments However this question can not be finally settled without direct data obtained from the canary

In the study referred to above Miller (1939) found that the increase in oxygen consumption after 0 8 mg/bird of thyroxine orally was 32 per cent The comparable parenteral dose was 0 15 mg/bird which gave an increase of 24 per cent The per

TABLE III Statistical evaluation (Student s t) of the differences between the increases in the length of circadian period no 0 after parenteral or oral administration of T3

Series used	Difference hours	t	DF	P
Parenteral PM versus oral AM	0 47	2 35	14	0 02 <p<0 05
Parenteral PM versus oral PM	0 47	2 94	44	0 001<p<0 01
Oral AM versus oral PM	0 00			NS

DF = degrees of freedom
NS = not significant

centage of thyroxine utilized after an oral administration in the English sparrow thus seems to be of the same magnitude as the one found for T3 in the precent experiments

The changes induced by T3 in the circadian rhythm were an increase in activity, decrease in rest and an increase in the length of the circadian period At the time of maximum change (in circadian period no 1) there was a high correlation between increase in activity and increase in period length However the time course of the change in activity and the change in circadian period were not parallel (Fig 1B) In circadian period number 0 (the period of administration) the increase in activity was roughly the same as in circadian period no 2 Circadian period no 0 was, however not increased while there was an increase of approximately half an hour of circadian period no 2 These data thus indicate that there was no absolute coupling between changes in activity (mainly due to change in time of roosting) and changes in the length of the circadian period (due to change in time of waking up) The same conclusion was reached after experiments with experimental dark periods after the expected waking up (Wahlstrom 1965b)

In the experiments with thyrotropine there was a slight increase in the length of circadian period no 0 (Fig 5) In the parenteral PM-experiments with T3 (fig 1C) there was also an increase in circadian period no 0 No comparable increase was found in the oral PM series with T3 (Figs 1B and 2) This difference was statistically significant (Table III) Probably effective levels of T3 were reached faster in the parenteral series In the oral AM series (Fig 1A) there was however no increase in length of circadian period no 0 although there was a fairly long time in which effective levels of T3 could have been reached during circadian period no 0 in these experiments The AM series differed from the PM series at the 5 per cent level (Table III) Furthermore no increase in circadian period no 1 was obtained in the oral AM series A probable explantation could be larger losses of oral T3 in the first part of the activity compared with the last part of it These early losses could also partly explain the regression between time from dose to "morning one" and increase in circadian period no 1 found in Fig 3A The reason why changes in activity in period no 1 did not show the same relation is uncertain There is some

evidence for a difference in the dose response curve for T3 between "activity" and 'circadian period' (Results part C)

After administration of T3 the changes in self selected circadian rhythm lasted for several days The weight changes induced by thyroxine discussed above also lasted several days No data on the half lives of thyroid hormones in adult canaries are available but in the 21 day-old chicken a value of 22.5 hrs was obtained for T3 as well as L-thyroxine by Tata and Shellabarger (1959) These data were obtained by measuring the residual radioactivity (^{131}I) by a total body ring γ counter Since the site of action of T3 in the present experiments is unknown the half life of T3 in the whole animal does not necessarily reflect the half life at the site of action Furthermore since there are fairly large species differences in the half life of triiodothyronine (Pitt Rivers and Tata 1959 p 130) it is an open question whether the long lasting effects seen here are due to direct effects of T3 or of intermediary steps are involved

The implication which the changes induced by thyroid hormones could have with regard to the modulation of the normal circadian rhythm will be discussed in another paper (Wahlstrom 1969)

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Three Different Systems of Monoamine-Storing Cells in the Gastrointestinal Tract of Fetal and Neonatal Rats

By

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Abstract

HÅKANSON, R., CH OWMAN and N-O SJÖBERG *Three different systems of monoamine-storing cells in the gastrointestinal tract of fetal and neonatal rats* Acta physiol scand 1969 75 213—220

The development of three monoamine-storing cell systems in the wall of the duodenum, glandular portion of stomach, and ileum has been followed histochemically in fetal and newborn rats, and compared with the number and characteristics of such cells in adults 5-Hydroxytryptamine can be demonstrated in mast cells and enterochromaffin cells already a few days [illegible]

already at a stage when the capacity to store monoamines has not yet developed in the enterochromaffin like cells, the system of monoamine cells quantitatively dominating in the [illegible]

5-Hydroxytryptamine (5 HT) in the rat is present mainly at three different sites in neurons of the central nervous system (Hillarp *et al* 1966), in nerve terminals and parenchymal cells of the pineal gland (Bertler *et al* 1964), and in the gastrointestinal tract, which contains more than half of total body 5-HT (Erspamer 1966) In this species, some of the gastrointestinal 5-HT is stored in mast cells located at the mucosal surface and in the submucosal and muscular layers (Enerback 1966, Håkanson *et al* 1967a) The remainder of bowel 5 HT is present in enterochromaffin cells which in the rat stomach are restricted to the region of the pyloric sphincter (Enerback 1966, Erspamer 1966, Håkanson and Owman 1966, Penttila 1966, Aures *et al* 1968a) Apart from these cells, the glandular portion of the gastric mucosa contains another extensive system of cells morpohologically similar to the enterochromaffin cells and capable of synthesizing and storing monoamines (Håkanson and Owman 1966 Håkanson *et al* 1967a) This system of "enterochromaffin like"

cells[1] represents the non mast cell store of gastric histamine (Håkanson and Owman 1967), but contains neither catecholamines nor 5 HT unless exogenous amine precursors are supplied (Håkanson and Owman 1966 Håkanson *et al* 1967a)

It was assumed that information on the development of the amine storing cells in the gastric mucosa would help to elucidate the physiological significance of these cells Previous reports have described the development of the cellular stores of gastric histamine and histidine decarboxylase in the fetal and neonatal rat (Håkanson *et al* 1967b, Aures and Håkanson 1968) In the present study, the development of the monoamine producing and monoamine storing capacity of the three systems of mast cells, enterochromaffin cells, and enterochromaffin-like cells was followed in various regions of the digestive tract of the rat, using a combination of histochemical and chemical methods

Material and methods

Albino rats from a closed colony were used The material includes fetal, newborn and adult animals

For the histochemical studies 75 fetuses (9 from each stage) were collected daily during 15—22 days (± 8 hrs) after mating as verified by the presence of sperms in stained vaginal smears Specimens from the glandular part of the stomach and from the duodenum and terminal ileum from 3 of the fetuses at each stage were incubated for 1 hr at 37° C in a Krebs Ringer solution A further 3 fetuses from the various stages were similarly incubated in the presence of 10 µg/ml of L DOPA (L 3 4 dihydroxyphenylalanine) The remaining fetuses served as untreated controls

36 newborn animals were taken at 0 1, 4 and 7 days *post partum* 4 of the animals from each of these stages were given 100 mg/kg of L-DOPA i p 1 hr before decapitation The remaining 5 animals from each group were included as untreated controls

11 adult animals of either sex weighing 150—200 g were used 5 of the animals were treated with L DOPA as above

Pieces from the glandular portion of the stomach from the duodenum and from the terminal ileum were taken from all animals and quenched to the temperature of liquid nitrogen and subsequently freeze dried The specimens were treated in formaldehyde gas from paraformaldehyde at +80° C during 1 hr (Falck 1962, Falck *et al* 1962 Corrodi and Hillarp 1963 1964, Corrodi and Jonsson 1967) In tissues from 2 of the non incubated control fetuses at each stage from 3 non injected newborns at each stage, and from 2 adults (non injected), formaldehyde-treatment was omitted All specimens were embedded in paraffin *in vacuo*, sectioned at 6 µ thickness and prepared for fluorescence microscopy Under the optical condi
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phosphate buffer (pH 7) The homogenates were used in a concentration of 10—100 mg tissue per ml Aliquots of the homogenates (usually 0 1 ml) were assayed for DOPA decarboxylase activity by a radiometric technique (Håkanson 1966) the enzyme activity was expressed per unit of tissue weight The fetal material was used for 6 determinations the newborn for 40 and the adult for 6 determinations

Dopamine was measured fluorometrically (Bertler *et al* 1958 Carlsson and Waldeck 1958) in the stomach wall of 6 newborn rats given L-DOPA (100 mg/kg i p) and 6 controls as well as of 4 adult rats given L-DOPA and 4 adult control animals In the determinations on newborn animals 3 whole stomachs were pooled for each assay

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Results

Mast cells Mast cells with a formaldehyde induced fluorescence could not be demonstrated until 19—20 days of gestation in sections from tissues, non incubated or incubated in Krebs Ringer solution alone At this stage, a few isolated large cells, having coarse cytoplasmic granules and emitting a specific, dirty yellow fluorescence of moderate intensity appeared in the submucosal layer This kind of yellow, formaldehyde induced fluorescence usually reflects the presence of 5 HT (Corrodi and Jonsson 1965), which is known to occur in mast cells of the rat (Lewis 1958) In all regions the number of mast cells increased during the subsequent stages. Even at the time of birth the cells had a remarkably coarse granulation as compared with adult mast cells The coarse granulation of the mast cells was apparent also 7 days after birth although the number and distribution of the cells more closely resembled that of the adult animals In preparations from adult animals the mast cells were located at various levels of the gastrointestinal mucosa as well as in the submucosa and in the muscle coat (Fig 1a)

In the fetal specimens incubated with L-DOPA, coarsely granulated, fluorescent mast cells could be identified at 18—19 days after mating The cytoplasmic granules emitted a green fluorscence, which is characteristic of DOPA and its catecholamine derivates (Falck and Owman 1965) The number and distribution of the mast cells with an induced green fluorescence resembled that of the 5 HT storing mast cells (which however, were not visible in the untreated animals until one day later)

During the following stages, both green and yellow fluorescent mast cells were visible in sections from tissues incubated in L-DOPA, or taken from animals injected with the amino acid In newborn animals treated with L-DOPA the green fluorescent mast cells were restricted to the mucosa, whereas all mast cells in the submucosa and muscular layers emitted a yellow light (Fig 1c) All cells of both types had a coarse and not very dense granulation This contrasted with the adult animals given L-DOPA in which both types of mast cells were recognized but those with a yellow fluorescence had now a more fine and dense granulation which is characteristic of mast cells elsewhere in the body

Enterochromaffin cells At 18—19 days *post coitum* fluorescent cells with a morphology characteristic of enterochromaffin cells, and emitting a yellow light presumably due to 5 HT, could be demonstrated in the mucosal epithelium of the duodenum (including the pylorus) and ileum The number of enterochromaffin cells was initially very small, the increase in later stages was particularly evident in the duodenum From birth on the number and fluorescence intensity of the enterochromaffin cells in the pyloric gland area was similar to that of adult animals In the oxyntic gland area yellow fluorescent enterochromaffin cells were usually absent at all stages including adult animals (Fig 1a) At most a few scattered cells were sometimes recognized in this region

No apparent change was observed in the colour of the emitted fluorescence of the enterochromaffin cells in preparations incubated in the presence of L-DOPA, or

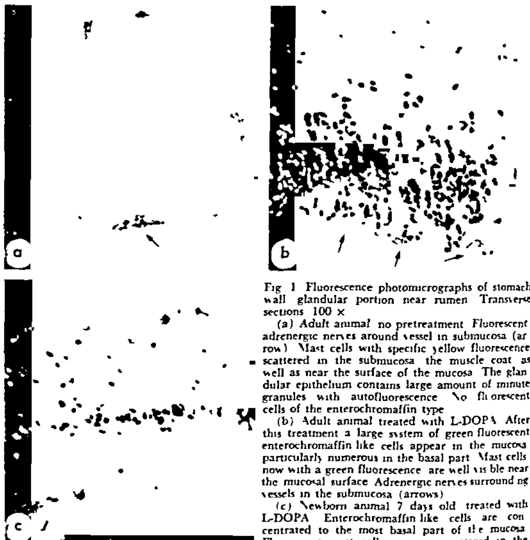


Fig 1 Fluorescence photomicrographs of stomach wall glandular portion near rumen Transverse sections 100 ×

(a) *Adult animal no pretreatment* Fluorescent adrenergic nerves around vessel in submucosa (arrow) Mast cells with specific yellow fluorescence scattered in the submucosa the muscle coat as well as near the surface of the mucosa The glandular epithelium contains large amount of minute granules with autofluorescence No fluorescent cells of the enterochromaffin type

(b) Adult animal treated with L-DOPA After this treatment a large system of green fluorescent enterochromaffin like cells appear in the mucosa particularly numerous in the basal part Mast cells now with a green fluorescence are well visible near the mucosal surface Adrenergic nerves surrounding vessels in the submucosa (arrows)

(c) Newborn animal 7 days old treated with L-DOPA Enterochromaffin like cells are concentrated to the most basal part of the mucosa Fluorescent mast cells are seen scattered in the rest of the mucosa the submucosa and the muscular coat

taken from animals treated with L-DOPA It cannot be excluded however that the high 5 HT content prevents the histochemical demonstration of small amounts of newly formed dopamine in these cells

Enterochromaffin like cells The properties of this large cell system in the oxyntic gland area of the gastric mucosa were originally studied in adult rats (Håkanson and Owman 1966 Håkanson *et al* 1967a Normally no monoamines can be demonstrated by fluorescence microscopy in the cells (Fig 1a) However they are capable of taking up and decarboxylating L-DOPA and 5 hydroxytryptophan to form dopamine and 5-HT, respectively which are then retained in the cytoplasm in high concentrations (Håkanson and Owman 1966 Håkanson *et al* 1967a) Direct com

parison of sections from the oxyntic gland area of animals pretreated with L-DOPA, first photographed in the fluorescence microscope and subsequently silver stained and re photographed, has demonstrated that the cells now storing dopamine correspond very well to the system of argyrophil (but non argentaffin) cells (Håkanson and Owman 1969) Moreover, these cells have been recognized as the major store of gastric histamine and histidine decarboxylase (Håkanson and Owman 1967, 1968 Aures *et al* 1968a)

Accordingly, this special type of cells could only be visualized in tissues after treatment with L-DOPA (Fig 1b and 1c) The green fluorescent flask shaped cells with the characteristic distribution within the mucosa of the glandular part of the stomach were first demonstrated at 22 days after mating *i e* immediately before birth

The number of the fluorescent cells in the gastric mucosa at birth and also one week postnatally (Fig 1c), was small compared with adult animals (Fig 1b)

Only scattered enterochromaffin like cells with a green fluorescence occurred in the duodenum. Neither at birth nor later were such cells found in the ileum

DOPA decarboxylase DOPA decarboxylase was followed in the stomach wall and in the intestines during fetal and postnatal development It should be noted that this enzyme is non specific and capable of decarboxylating a number of aromatic amino acids including 5 hydroxytryptophan (Rosengren 1960, Lovenberg *et al* 1962) Thus the enzyme is different from the more specific mammalian histidine de carboxylase (Håkanson and Owman 1966 Aures *et al* 1968a) The DOPA de carboxylase activity was quite high 18 days after mating (at least 50 per cent of the adult value) Adult levels were reached at birth after which the enzyme activity remained at a fairly constant level In the stomach wall of newborn rats treated with L-DOPA, 3 1 μg/g (mean value) dopamine could be demonstrated one hour after the injection The corresponding value for gastric dopamine in adult animals was 4 3 μg/g Non measurable amounts were found in the untreated young and adult control animals

Discussion

The gastrointestinal tract of the rat contains two kinds of mast cells Histochemically both populations of mast cells have been found to store 5 HT (Enerback 1966 Håkanson *et al* 1967a) as well as histamine (Håkanson and Owman 1967 Thunberg 1967) One type is located in the submucosa and the muscular coat and has a characteristic dense and fine granulation. The other type that is found in the bowel mucosa in the stomach characteristically near the surface has a more sparse granulation and the granules are quite coarse The histological properties of the mucosal mast cells differ in other respects from ordinary mast cells and they have been classified as atypical Characteristically, these 'atypical mast cells are resistant to the degranulation effect of 48/80 (Enerbäck 1966 Aures *et al* 1968b) The two populations of mast cells differ also in that administration of L-DOPA

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The Evolution of Vascular Smooth Muscle Responses to Histamine and 5-Hydroxytryptamine

I. Occurrence of stimulatory actions in fish

By

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Abstract

Reite, O. B. *The evolution of vascular smooth muscle responses to histamine and 5-hydroxytryptamine.* I. Occurrence of stimulatory actions in fish. Acta physiol. scand. 1969. *75.* 221—239.

In an attempt to trace the phylogenetical origin of stimulatory (constrictive) and inhibitory (dilatatory) actions of histamine and 5-hydroxytryptamine on mammalian vascular smooth muscle, the vascular responses to these drugs were studied in jawless vertebrates and fish. Effects were recorded as blood pressure changes in dorsal and ventral aorta after intravascular administration of drugs, and as resistance changes in artificially perfused branchial and systemic blood vessels after administration into the perfusion circuit. The reliability of experimental procedure and recording technique was tested by observing the presence of responses to adrenaline, noradrenaline or acetylcholine. Pharmacological blocking agents were used to dissociate between specific and unspecific actions. In jawless vertebrates, the vascular effects of histamine and 5-hydroxytryptamine seemed to reflect unspecific actions on adrenergic receptor mechanisms. The effects in cartilaginous fish were slight. Characteristic effects, produced by specific stimulatory actions on vascular smooth muscle, appeared in higher bony fish. Inhibitory actions were not revealed in any species. By comparing the results obtained in intact animals with those from perfused preparations it may be concluded that stimulatory actions of 5-hydroxytryptamine appear as a constrictor response in the branchial blood vessels of teleosts while the stimulatory actions of both histamine and 5-hydroxytryptamine are fully developed and produce vasoconstriction in all studied sections of the vascular system in lungfish.

The vast majority of available information on the significance of histamine and 5-hydroxytryptamine in vertebrate biology is based on experiments in mammals. Widely distributed and pharmacologically potent as they are, both substances have been considered as potential mediators in numerous physiological processes (Feldberg and Schilf 1930, Erspamer 1954, Brodie, Pletscher and Shore 1955, Rocha e Silva 1955,

[1] Part of the experimental work was carried out during short stays at the Biological Station of the University of Oslo, Drøbak, and at the Friday Harbor Laboratories of the University of Washington, Friday Harbor, Washington, U.S.A.

Code 1956, Page 1958, Kahlson 1960, Schayer 1962) Their most conspicuous actions are those on smooth muscles (Dale and Laidlaw 1910—11, 1911—12, Feldberg and Schilf 1930, Page 1958, Erspamer 1961) The direct actions of either substance on the smooth muscles of the mammalian vascular system comprise a stimulatory and an inhibitory component, rendered manifest as vasoconstriction and vasodilatation, respectively (Dale and Laidlaw 1918—19, Dale and Richards 1918—19, Feldberg and Schilf 1930, Rapport, Green and Page 1948, Erspamer 1961) The relative importance of these two components is influenced by the prevailing vascular tone, and species differences are evident (Dale and Richards 1918—19, Feldberg and Schilf 1930, Haddy, Gordon and Emanuel 1959, Erspamer 1961) However, while other biologically occurring vasoactive substances like adrenaline, noradrenaline and acetylcholine can be functionally classified as hormones or neurohumors, the participation of histamine and 5 hydroxytryptamine in normal vascular phenomena remains uncertain

Since extensive studies in mammalian species have failed to explain the functional significance of histamine and 5 hydroxytryptamine in relation to the vascular system, a broader background of knowledge seems required I have therefore undertaken a series of experiments designed to elucidate the general pharmacological features of histamine and 5 hydroxytryptamine in vertebrates, with the main interest centered on their actions on vascular smooth muscle A phylogenetical approach was chosen The two components in the characteristic vascular responses to histamine and 5-hydroxytryptamine in mammals were looked upon as separate qualities, and were individually traced as a stimulatory and an inhibitory vascular response among the relatives of mammalian ancestors, as represented by the lower vertebrates Starting with the most primitive species changes in response pattern which have occurred during evolution of higher forms were systematically studied and related to known functional and structural changes within the vascular system

Several investigative procedures were applied Systemic arterial blood pressure responses in intact animals were recorded during intravascular administration of histamine and 5 hydroxytryptamine The action of histamine and 5 hydroxytryptamine on artificially perfused blood vessels was also investigated Effects of released endogenous amines were studied by injection of agents which deplete tissue stores The responses of the different circulatory systems to adrenaline, noradrenaline and occasionally acetylcholine were studied for comparison Appropriate blocking agents were applied for studying the specificity of receptors Tissue levels of histamine and 5 hydroxytryptamine were determined by spectrofluorometrical assay techniques and histochemical localization was attempted

Information has been gained from some 30 European, American and African species, representing every subclass of living lower vertebrates This report deals with observations made in jawless vertebrates cartilaginous fish and higher bony fish which—as opposed to other vertebrates—have in common that sympathetic nervous control of vascular smooth muscle has not been demonstrated in any species Results obtained in amphibians and reptiles will be treated separately

TABLE I Experimental animals

Species	Number of animals	Approximate body weights* (kg)
Jawless vertebrates		
Atlantic hagfish (*Myxine glutinosa*)	15	0 04—0 08
Pacific hagfish (*Polistotrema stouti*)	4	0 05—0 06
Cartilaginous fish		
Pacific dogfish (*Squalus suckleyi*)	8	2—4 5
Basking shark (*Cetorhinus maximus*)	2	600—1200
Big skate (*Raja binoculata*)	3	9—14
Ratfish (*Hydrolagus colliei*)	4	0 6—1 2
Rabbit fish (*Chimaera monstrosa*)	7	0 04—0 1
Higher bony fish		
Bichir (*Polypterus senegalus*)	3	0 1—0 25
Rainbow trout (*Salmo irideus*)	8	0 6—0 9
Common eel (*Anguilla anguilla*)	9	0 4—0 8
Cultus cod (*Ophiodon elongatus*)	3	6—10
Atlantic cod (*Gadus morhua*)	12	0 5—3 5
Cabezon (*Scorpaenichthys marmoratus*)	2	3—4
African lungfish (*Protopterus aethiopicus*)	6	2 5—5 5

* Weights estimated after weighing of one or a few animals from each species

Material and Methods

Experimental animals The studied species are listed in Table I They are chosen as representative not only of the different classes but also of the different forms within each class Marine fish were caught by trawl or bow-net A few days elapsed between catching and performance of experiments In the meantime the fish were maintained without food in large holding tanks with constantly aerated circulating sea water at a temperature of about 8° C The two specimens of basking shark which had been captured in a net were brought ashore alive and their tissues studied shortly afterwards Eels were acquired similarly to marine fish but were transferred to fresh water of 8—10° C and occasionally kept for several weeks before being used The rainbow trout were obtained from a fish hatchery and were also kept at 8—10° C in a fresh water aquarium. Hagfish, bichir and lungfish were acquired through biological supply houses Cyclostomes were maintained as indicated for marine fish Bichir and lungfish were kept in fresh water at 20—25° C The experiments were performed at different seasons of the year

Surgical procedure Fish were anesthetized by transference to a solution made up in a shallow container by adding tricaine methane sulphonate (MS 222 Sandoz) to water from their aquarium Using a concentration of anesthetic of 0 01—0 05 per cent satisfactory immobilization was easily achieved The fish were then immediately placed ventral side up in a trough or on a wooden board and secured to the underlayer by crosswise straps Oxygen was supplied by irrigation of the gills with pure aerated water taken from the aquarium of the same species of fish or similar aerated water containing the anesthetic Both fluids were pumped slowly through a spray nozzle placed in the mouth of the fish and light anesthesia was maintained by alternating the fluids as indicated by appearance and cessation of muscular movements Incisions for access to the blood vessels were made along or near the ventral midline One vein and one artery were always dissected free and cannulated with polyethylene catheters filled with heparinized physiological solution the vein for intravenous administration of

Cannulation of one of
evel of the second or
Dorsal aortic blood
or mesenteric artery,
advancing the catheters towards the dorsal aorta In rainbow trout, instead of measuring pressure in the aorta, ventricular blood pressure was obtained by puncturing the ventricle with a hypodermic needle connected to a ca...

surgical procedure rarely exceeded 30—40 min

Blood pressure recordings In a few species of fish, the experiment was started while the animal was still anesthetized and in the same restrained position as during implantation of catheters By means of a hypodermic needle and a three-way stop-cock, the arterial catheter was connected to a recording system, consisting of a Statham pressure transducer (Model P 23 Db, P 23 Gb or P 23 AA), combined with either a Beckman-Offner or a Sanborn dynograph Blood pressures were recorded continuously during administration of the different pharmacological agents The initial dose levels were usually in the range of 1—10 μg, but if no response was obtained the subsequent doses were increased Both intra-arterial and intravenous administration were tested, and the agents were given in varying sequences This was the sole method by which *in vivo* experiments were performed in hagfish, skate, rainbow trout and eel, *which were all sacrificed upon completion of the acute procedure Specimens from the other species of fish were not studied* ...
was completed, the free ends o
and the fish returned to their
In the other species, recovery
freely Later the same day, and on successive days, the fish were transferred individually to a smaller tank containing aerated water pumped from the aquarium The toothpicks were removed from the catheter openings and replaced with hypodermic needles connected to three way stop-cocks. Similar continuous recordings of blood pressure during intravenous administration of pharmacological agents were then performed, using the same recording device as for anesthetized fish Lungfish were studied both in the fully conscious state and before complete recovery from anesthesia. Most of the time during an experiment the fish under study rested quietly near the bottom of the small tank, breathing and slow movements of fins and tail being the only signs of activity However now and then it would swim around the tank a couple of times before resuming the resting position Movement of this kind did not interfere with the experiment as the pressure transducer was placed in the centre of the tank, close to the water surface and the catheters from the fish had a free length of 60—80 cm Differences in catheter lengths had only negligible influence on the pressure recordings. Some of the more sluggish species i e, cabezon and cultus cod were sometimes left in their aquarium throughout the study the respective catheter ends simply being hooked up for blood pressure recording and injection each time an experiment was to be performed After completion of the study, the catheter openings were closed and the fish released or returned to their aquarium In all specimens repetitive experiments were made over a period of 1—4 days During this time the fish showed no ill effects from the chronically indwelling catheters

Perfusion experiments The systemic blood vessels of one or more species from each subclass were perfused through the dorsal aorta with physiological solutions utilizing an adjustable peristaltic pump (Model 1203 Harvard Apparatus Co), and the branchial blood vessels were similarly perfused through ventral aorta During perfusion the blood vessels were usually left *in situ* and the corresponding efferent arteries or veins cut open to secure free outflow Branchial blood vessel perfusion was occasionally performed in the excised gill apparatus, but excision had no influence on the response pattern Due to the large size of the basking shark the only study performed in this species was perfusion of the coronaries and one of the main arteries of the anal fin A polyethylene catheter connected the latex tubing of the pump with the artery to be perfused The perfusion pressure was continuously monitored from this tubing with a transducer and recording device similar to that used for the blood pressure measurements Initially during an experiment, the perfusion pump was adjusted to the flow rate required to maintain a level of perfusion pressure within the range of normal blood pressures in fish. As a rule, the same constant flow rate was used throughout an experiment Single doses ... other pharmacological agents dissolved in small volumes
mp with the artery
were added to the
perature of the per-

fusion fluid was kept at 20—22° C for bichir and lungfish and at 5—12° C for the other species The perfusion was started shortly after the fish had been removed from their aquarium and sacrificed

Pharmacological agents Physiological solutions were made from a sterile stock solution containing 0 86 % NaCl 0 03 % KCl and 0 05 % $CaCl_2$ Before use this solution was modified with respect to inorganic salts and urea to fulfil the different osmotic requirements of hagfish cartilaginous fish and higher bony fish (Lockwood 1961) For hagfish one part of the stock solution was mixed with two parts of sea water For use in cartilaginous fish 0 6 % NaCl and 2 5 % urea were added to the stock solution The solution for lungfish and bichir was prepared by dilution of six parts of stock solution with one part of distilled water In the other species the stock solution was used without modification

The pharmacological agents tested or used in complementary studies comprised histamine phosphate 5 hydroxytryptamine creatinine sulfate (serotonin creatinine sulfate) adrenaline hydrochloride noradrenaline bitartrate, acetylcholine chloride compound 48/80 (condensation product of p-methoxyphenethylmethylamine with formaldehyde conf Paton 1951), polymyxin B sulfate protamine sulfate reserpine, pyrilamine maleate, chlorpheniramine maleate methysergide maleate (UML-491), dihydroergotamine methanesulfonate phentolamine methanesulfonate propranolol hydrochloride atropine sulfate and tubocurarine chloride Doses of histamine 5 hydroxytryptamine adrenaline and noradrenaline are expressed as weight of free base polymyxin B as antibacterial assay units and the other agents in terms of weight of the compounds in the form listed above The different agents were dissolved in physiological solution just prior to the experiments The volume of a single intravascular injection ranged from 0 1 ml in the smallest specimens to 2 ml in the largest During artificial perfusion volumes of 0 05—0 5 ml were introduced into the perfusion system Effects caused by the injection *per se* were checked by administration of the same volume of physiological solution

Spectrofluorometric assay of histamine and 5-hydroxytryptamine Tissues were excised from freshly killed specimens and homogenized in about ten times their own volume of 0 4 N perchloric acid The extracts were assayed for histamine according to the method of Shore Burkhalter and Cohn (1959) utilizing a Farrand spectrofluorometer combined with a Varian recorder, and taking the same precautions as mentioned elsewhere (Reite 1965) In order to use the same perchloric acid tissue extract for the assay of 5-hydroxytryptamine 4—10 ml samples were pipetted off and cooled in an ice bath The perchlorates were then precipitated as potassium perchlorate by adding drops of 5 M K_2CO_3 to pH 4 After centrifugation the 5-hydroxytryptamine content of the supernatant was determined in accordance with the procedure described by Udenfriend (1964) Spectrofluorometer and recording device were the same as used for the assay of histamine The fluorescence of 5 hydroxytryptamine in strongly acid solution was utilized in the routine determinations but presence of the proper fluorescence characteristics was also checked by scanning of the activation and fluorescence spectra in slightly acid solutions of the purified extracts The content of both amines was calculated as micrograms of the base per gram of fresh tissue

Histochemical localization Attempts to localize cellular or intercellular depots of histamine were made in tissues from the lungfish The method was based on observation of the fluorophore formed by interaction of histamine with o-phthalaldehyde the same principle as that applied in the spectrofluorometrical assay of histamine All studies were done with a Leitz Ortholux microscope equipped for fluorescence microscopy Except that tissue spreads had been air dried at 30—40° C before use the preparation technique and filter arrangement described by Juhlin and Shelley (1966) were followed starting with incubation at temperatures of 20—50 C in physiological solution buffered to pH 11 and using xylene as solvent for the o-phthalaldehyde Observations on the tissue mast cells in lungfish were made by ordinary light microscopy of fresh tissue spreads after direct staining with 0 1 % toluidine blue in physiological solution and after similar staining of tissue spreads previously used for the histochemical localization of histamine

Results

Resting heart rate and blood pressure

Values obtained during continuous recording over 5—15 min periods prior to drug administration are listed in Table II In unanesthetized fish, both heart rate and blood pressure showed spontaneous variations which were absent or only slight in anesthetized specimens As the experiment proceeded these variations disappeared and heart rate and blood pressure usually increased The heart rate in anesthetized

TABLE II Heart rate and blood pressure*

Species	Heart rate (beats/min)	Blood pressure (mm Hg)		Site of recording	Type of anesthesia
		Systolic	Diastolic		
Atlantic hagfish	27—32	9—13	5—8	Ventral aorta	MS 222
Pacific hagfish	24—30	10—15	7—11	Ventral aorta	MS 222
Pacific dogfish	13—20	28—32	18—22	Ventral aorta	None
		21—25	14—17	Dorsal aorta	None
Big skate	10—14	18—24	10—16	Ventral aorta	MS 222
Ratfish	18—26	22—28	15—19	Ventral aorta	None
		16—21	12—15	Dorsal aorta	None
Rainbow trout	55—64	45—52	0	Heart ventricle	MS 222
Common eel	39—46	33—42	26—34	Ventral aorta	MS 222
Cultus cod	32—38	40—46	36—41	Dorsal aorta	None
Atlantic cod	30—43	42—55	28—39	Ventral aorta	None
		30—38	24—31	Dorsal aorta	None
Cabezon	37—40	37—42	34—38	Dorsal aorta	None
African lungfish	24—29	32—41	24—34	Dorsal aorta	None

* Figures for heart rates are based on counting over several one minute periods, while those for blood pressure represent the highest and lowest levels within the period of observation Ambient water temperature was 20—25 C for lungfish and 7—12 C for the other species

fish was stable or showed a decrease in the course of an experiment There often appeared to be a parallel relationship between changes in heart rate and blood pressure

Effects of pharmacological agents

Jawless vertebrates Intravenous administration of histamine (5—25 μg), 5-hydroxytryptamine (1—25 μg) adrenaline (1—10 μg), noradrenaline (1—10 μg) or compound 48/80 (25—50 μg) had no profound vascular effects in any of the two species of hagfish There was a transient change in the frequency of the heart and in blood pressure associated with each injection In both, an increase was usually observed (Fig 1), but at the beginning of an experiment a subsequent decrease in blood pressure was quite common The response seemed more dependent on the volume injected than on the nature of the pharmacological agent However, when an experiment had lasted for 1—2 hrs ,the heart rate slowed and in this situation both adrenaline and noradrenaline had a marked accelerating effect on the heart and a long-lasting increase in blood pressure occurred concomitantly The changes brought about by the catecholamines were greater than those following histamine 5-hydroxytryptamine or control injections of saline although it was noted that all injections now produced a more pronounced increase in the frequency of the heart and in blood pressure than when the initial heart rate was higher

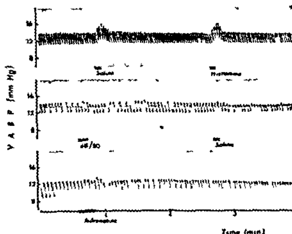


Fig 1 Recording of ventral aortic blood pressure (V.A.B.P.) in the Pacific hagfish. Except for pressure changes due to the i.v. injected volume of fluid (physiological solution, in figures referred to as saline) there was no clear response to histamine (25 μg) or compound 48/80 (50 μg), while adrenaline (5 μg) caused an increase in heart rate and blood pressure when the prevailing heart rate was slow, as shown here

The systemic and branchial blood vessels of the Atlantic hagfish were perfused through dorsal and ventral aorta respectively, and doses of histamine, 5-hydroxytryptamine adrenaline and noradrenaline similar to those used in the intact animal were tested The resistance of systemic blood vessels was consistently increased by adrenaline and noradrenaline, and either showed no change or a slight increase after large doses of histamine and 5-hydroxytryptamine Acetylcholine (1—5 μg) caused increased resistance in systemic as well as in branchial blood vessels A rapid but transient increase in resistance which appeared in the branchial system immediately following administration of acetylcholine, was apparently produced by a twitch-like contraction of striated muscles, and was absent when tubocurarine (5 μg/ml) had been added to the perfusion fluid The branchial blood vessel responses to histamine 5-hydroxytryptamine adrenaline and noradrenaline were similar to each other and revealed the presence of both an inhibitory and a stimulatory component (Fig 2 and 3) Early in an experiment the inhibitory action producing decreased vascular resistance, was dominant (Fig 2) This picture gradually changed The change started with the appearance of a triphasic response a transient pressure increase which was followed first by a decrease and then by a more sustained increase Later the decrease became less pronounced and finally the typical response pattern was an increase in perfusion pressure (Fig 3) Both the inhibitory and the stimulatory components of the response to histamine 5 hydroxytryptamine and catecholamines were unaffected by chlorpheniramine (2—5 μg/ml) and methysergide (3—5 μg/ml), which are recognized blocking agents for histamine and 5-hydroxytryptamine respectively The same response also remained after atropine (3 μg/ml) and after curarization of the preparation by tubocurarine (5 μg/ml) However the stimulatory component of the response to the biogenic amines was completely abolished by the α-adrenergic blocking agents phentolamine (4 μg/ml) and dihydroergotamine (4 μg/ml) whereas the inhibitory component was abolished by the β-adrenergic blocking agent propranolol (3—5 μg/ml) When one of the components was depressed by

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Ratfish	18—26	22—28	15—19	Ventral aorta	None
		16—21	12—15	Dorsal aorta	None
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Common eel	39—46	33—42	26—34	Ventral aorta	MS 222
Cultus cod	32—38	40—46	36—41	Dorsal aorta	None
Atlantic cod	30—43	42—55	28—39	Ventral aorta	None
		30—38	24—31	Dorsal aorta	None
Cabezon	*37—40*	*37—42*	*34—38*	*Dorsal aorta*	*None*
African lungfish	24—29	32—41	24—34	Dorsal aorta	None

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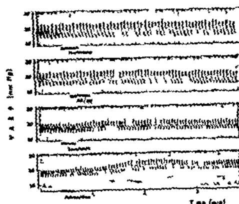


Fig 4

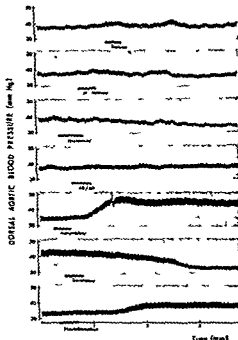


Fig 5

Fig 4 Ventral aortic blood pressure recordings from the Pacific dogfish No effects of i v administration of histamine (100 µg) or compound 48/80 (100 µg) but a small pressure increase after administration of 5-hydroxytryptamine (serotonin 10 µg) and a marked increase after adrenaline (10 µg)

Fig 5 Recording of dorsal aortic blood pressure in the cabezon Pharmacological agents were given i v There was no effect of physiological solution (saline) or compound 48/80 (50 µg) The upper of the two recordings obtained during injection of histamine shows a small decrease in pressure after 100 µg while the subsequent recording shows that this decrease was augmented by increasing the histamine dose to 200 µg Adrenaline (15 µg) and noradrenaline (10 µg) produced a marked and long lasting increase in pressure The pressure was decreased by 5 hydroxytryptamine (serotonin, 10 µg) Note the disappearance of spontaneous pressure fluctuations as the experiment proceeded

(2 500—15 000 units) and protamine (0 5—1 mg) were performed in dogfish and were also without effect Typical blood pressure responses recorded from the ventral aorta of dogfish are depicted in Fig 4 Route of administration or sequence in which the drugs were given did not alter the response pattern

Perfusion of the coronary arteries and the arteries of the anal fin of the basking shark revealed no effect of histamine (10—40 µg) or 5 hydroxytryptamine (5—25 µg) nor did perfusion of dorsal aorta in rabbit fish A weak pressure response was obtained in all these preparations following administration of adrenaline and noradrenaline in doses of 10—20 µg

Higher bony fish The perfused systemic blood vessels of the bichir responded to adrenaline (10—20 µg) and noradrenaline (10—20 µg) by increased resistance, whereas similar doses of histamine and 5 hydroxytryptamine were without effect

In teleosts there was a general conformity in the nature of the blood pressure responses to the different drugs, but response magnitudes varied considerably. Recordings obtained from one of the more sensitive species, the cabezon, are shown in Fig. 5. The cabezon did not respond to 10—50 μg of histamine, but 100 μg given i.v. produced a slight decrease in dorsal aortic blood pressure. This decrease was augmented by increasing the i.v. dose to 200 μg. It developed slowly and was accompanied by decreased pulse pressure. Administration of histamine into dorsal aorta of the cabezon gave a weaker response than via the intravenous route. No measurable responses to histamine (10—200 μg) were found in cod, rainbow trout, eel or cultus cod. In the latter, histamine doses as high as 1 mg were tested without effect. Intravenous or i.a. injection of 5-hydroxytryptamine (2—30 μg) caused a decrease in both pressure level and pulse pressure in dorsal aorta of all species. Compared to i.v. administration, injection into dorsal aorta gave a delayed response. The effect of 5-hydroxytryptamine on ventral aortic blood pressure was studied in cod and eel and showed a marked increase which rapidly reached its maximal amplitude and persisted for several minutes. Systolic ventricular blood pressure in rainbow trout was also increased after injection of 5-hydroxytryptamine. Adrenaline and noradrenaline (2—20 μg) induced long lasting elevation of blood pressure in both dorsal and ventral aorta of all the studied species. No effect was obtained with compound 48/80 (25—500 μg), protamine (0.5—2 mg) or polymyxin B (2,500—15 000 units). The two latter agents were tested only in cultus cod.

During perfusion of the systemic blood vessels of cod and eel a slight increase in resistance was occasionally observed following administration of large doses of histamine (40—50 μg) and 5-hydroxytryptamine (20—30 μg). Continuous infusion of these agents by adding 1—2 μg/ml to the perfusion fluid similarly produced only negligible effects. Acetylcholine (10—30 μg) caused a more pronounced increase in resistance. The resistance remained unchanged after compound 48/80 (50—100 μg). After adrenaline and noradrenaline (2—10 μg), the resistance across the systemic vascular bed was always markedly increased. The perfused branchial blood vessels of these species showed a constrictor response to the same doses of 5-hydroxytryptamine and acetylcholine as those tested on the systemic vascular bed, but also showed a high sensitivity to the drugs, especially to 5-hydroxytryptamine (Fig. 6). In the eel the largest histamine doses administered (40—50 μg) produced a decrease in branchial resistance, whereas after a similar dose in the cod a slight increase was the only effect observed. Histamine at lower dose magnitudes (20—30 μg) in both species either had no effect or caused a negligible increase in branchial resistance. Doses of adrenaline and noradrenaline, which regularly increased the resistance of the systemic vascular bed in the two species, produced decreased resistance when tested on the branchial blood vessels. The decrease was occasionally preceded by a transient increase. Typical responses to adrenaline, histamine, acetylcholine and noradrenaline in the eel are shown in Fig. 7. The branchial response to 5-hydroxytryptamine was blocked by methysergide (2 μg/ml) but remained after atropine (3 μg/ml). The inhibitory action of histamine was unaffected by antihistamine (pyrilamine 10 μg

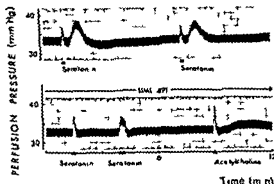


Fig 6

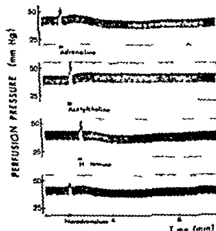


Fig 7

Fig 6 Perfusion of the branchial blood vessels in the Atlantic cod Effects of 5 hydroxytryptamine (serotonin) The upper recording shows the pressure response to successive injections of 2 µg and 1 µg respectively indicating a pronounced increase in resistance The lower recording was obtained shortly afterwards in the same preparation but here methysergide (UML-491 2 µg/ml had been added to the perfusion fluid causing blockade of the response to 5 hydroxytryptamine injections (2µg and 10 µg) while the normal pressure response to acetylcholine (10 µg) was unaffected

Fig 7 Branchial blood vessel responses obtained during a perfusion experiment in the eel The recordings show the resistance changes resulting from injections of adrenaline (5 µg) acetylcholine (10 µg histamine (50 µg) and noradrenaline (3 µg)

ml The β adrenergic blocking agent propranolol (4 µg/ml on the other hand effected a blockade of the inhibitory actions of adrenaline and noradrenaline as well as of large doses of histamine and revealed the presence of an otherwise masked stimulatory response to the catecholamines

In the intact lungfish the effect of 2—20 µg of histamine 5 hydroxytryptamine adrenaline or noradrenaline was a rise in dorsal aortic blood pressure The pressor action of histamine was of short duration, being succeeded by a fall down to or slightly below pre injection levels Results obtained during recovery from anesthesia are shown in Fig 8 Additional behavioural responses including respiratory efforts disturbed recording of the effects of histamine and 5 hydroxytryptamine on the blood pressure in fully conscious specimens although it was evident that the nature of the responses remained the same

The blood vessels of the hind part of the lungfish were perfused through dorsal aorta Both histamine and 5 hydroxytryptamine elicited a marked increase in perfusion pressure even when administered in rather low doses (2—5 µg) Their effects were depressed or abolished by pyrilamine 10—15 µg/ml and methysergide (3 µg/ml respectively The perfusion pressure was also increased by adrenaline, noradrenaline and acetylcholine in doses of 5—20 µg Compound 48/80 (20—40 µg) poly

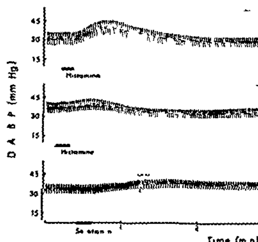


Fig 8

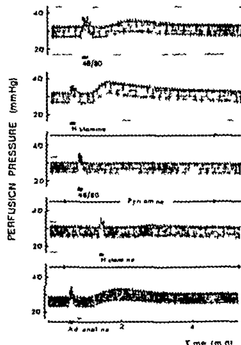


Fig 9

Fig 8 Recording of dorsal aortic blood pressure (D A B P) in lungfish during recovery from anesthesia The pressure increase produced by i v injection of 10 µg histamine (upper recording) showed a rapid decline The following recording shows that when the initial blood pressure was elevated (in this case by previous administration of adrenaline) the pressure response to histamine (10 µg) was less marked but the subsequent rapid decline was present The pressure increase caused by 5 hydroxytryptamine (serotonin 5 µg) was of rather long duration

Fig 9 Perfusion of the isolated hind part of the lungfish The two upper recordings show the increase in perfusion pressure induced by compound 48/80 (40 µg) and histamine (? µg) respectively During the subsequent recordings pyrilamine (15 µg/ml) had been added to the perfusion fluid The responses to compound 48/80 (40 µg and histamine (20 µg) were now abolished or strongly depressed while there was a normal response to adrenaline (15 µg

myxin B (2 500 units) and protamine (1 mg) all induced an increase in perfusion pressure comparable to that produced by histamine and this response was prevented by adding either pyrilamine (15 ug/ml) or chlorpheniramine (4 ug/ml) to the perfusion fluid Reserpine (100 ug) had no effect Typical responses to some of the tested agents are depicted in Fig 9

Tissue levels of histamine and 5 hydroxytryptamine

Histamine and 5 hydroxytryptamine were assayed in skin from Atlantic hagfish rabbit fish and basking shark in mesentery from bichir and in skin muscle gills stomach and intestine from cod eel and rainbow trout Except for the stomachs none of these tissues showed histamine levels which exceeded 1 7 ug/g Most values were in a range well below 1 µg/g In the stomach the histamine determinations

gave results ranging from 6.5—19.8 μg/g, with large individual variations. Assay for histamine in skin and muscles from cod and eel killed within a few minutes after injection of large doses intravenously, showed elevated tissue levels. If large doses of 5-hydroxytryptamine had been administered similarly, measurable amounts of this substance were also found in the tissue extracts, but otherwise no 5-hydroxytryptamine seemed present in any of the tissues studied. With the technique applied in the present work, it is implied that the 5-hydroxytryptamine levels were below 0.2 μg/g tissue, but in a few cases where small amounts of tissue were available, levels as high as 0.5 μg/g may have escaped detection.

In the lungfish, assay was performed in samples from skin, muscle, tongue, liver, lung and mesentery. 5-hydroxytryptamine was apparently absent in all these tissues. Histamine levels of 6.4—18.6 μg/g tissue were found in skin, tongue, lung and mesentery. The contents of lung and tongue were in the lower part of this range, those of skin and mesentery in the upper. Muscle and liver showed a histamine content of 1.2—3.5 μg/g. Small pieces of skin incubated for 1 hour at 22° C in physiological solution containing compound 48/80 (1 μg/ml) showed a histamine loss which amounted to about one fifth of the total content, and measurable quantities of histamine could be recovered from the incubation fluid. Skin incubated similarly in pure physiological solution showed negligible loss of histamine. Pieces of fresh mesentery also showed a negligible loss of histamine when incubated in physiological solution, whereas such incubation of mesentery previously exposed to freezing and thawing resulted in loss of most of the histamine content within an hour.

Histochemical localization of histamine

In tissue spreads from the mesentery of lungfish prepared for histochemical localization of histamine, a diffuse yellow fluorescence was observed in some areas within each preparation. This fluorescence became still more diffuse during the period of observation and eventually faded. Subsequent staining with toluidine blue gave no conclusive results as to the cellular origin of the fluorescing material. A few cells in or near small blood vessels showed a distinct but weak fluorescence. Staining with toluidine blue revealed that these cells had a large round nucleus, surrounded by a rather thin layer of cytoplasm with numerous granules which stained metachromatically. The cells were probably blood basophils. Their surface was usually rugged, and some of the granular material had apparently become extruded from the cells and was sticking to their exterior. The mast cells in the same tissues had become degranulated. Fresh preparations directly stained with toluidine blue showed intact tissue mast cells when studied immediately upon staining, but the mast cells gradually underwent degranulation. A metachromatic halo appeared in the vicinity of the degranulated cells, indicating partial solubility of the granular material in water. Incubation of tissues in physiological solution seemed to have negligible effects on the mast cells. Exposure to compound 48/80 or freezing and thawing, on the other hand, caused rapid degranulation.

Discussion

Interpretation of results obtained by blood pressure recording in dorsal or ventral aorta are complicated by the fact that branchial and systemic blood vessels in fish are coupled in series Assuming a constant cardiac output an increase or decrease in dorsal aortic blood pressure is suggestive of constriction or dilatation of systemic blood vessels respectively but leaves open the question of possible branchial resistance changes Similarly the only conclusion permissible from observation of changes in ventral aortic blood pressure is that the total resistance of branchial and systemic blood vessels is altered If the pharmacological agents also affect cardiac output through direct or indirect actions on the heart the interpretations will become further complicated Both blood pressure changes and resistance changes in artificially perfused systemic and branchial blood vessels have therefore to be considered

In intact fish the only recorded vascular effects of histamine were changes in dorsal aortic blood pressure There was a slight decrease following administration of high doses in the cabezon and a marked increase after all tested doses in lungfish Vascular effects of 5 hydroxytryptamine were more commonly encountered Independent of route of administration of this drug the systolic ventricular blood pressure of rainbow trout was increased and the blood pressure in ventral aorta showed a small increase in ratfish and dogfish and a striking increase in cod and eel Dorsal aortic blood pressure was consistently decreased by 5 hydroxytryptamine in teleosts and increased in lungfish Responsiveness to adrenaline and noradrenaline was also observed in species which were apparently insensitive to histamine and 5 hydroxytryptamine indicating that experimental procedure and recording technique were adequate

In the branchial blood vessels of hagfish both histamine and 5 hydroxytryptamine mimicked the actions of adrenaline and noradrenaline the stimulatory and inhibitory actions being blocked by α and β adrenergic blocking agents respectively Since the blocking agents for histamine and 5 hydroxytryptamine did not affect either of these responses it seems justified to consider the stimulatory as well as the inhibitory actions of histamine and 5 hydroxytryptamine on this preparation as unspecific responses brought about by release of endogenous adrenaline or noradrenaline or more likely by triggering of adrenergic receptor mechanisms The instability of the obtained responses as reflected by a change from mainly inhibitory to mainly stimulatory actions on branchial blood vessels while the systemic response to the same agents was absent or consistently stimulatory may explain why the blood pressure responses in hagfish were somewhat difficult to interpret

Perfusion experiments in cartilaginous fish gave no indication of any response to histamine and thus confirmed the blood pressure measurements The results obtained with 5 hydroxytryptamine were also negative However perfusion of branchial blood vessels from these fish was not performed and an increase in ventral aortic blood pressure as observed in dogfish and ratfish with no pressure change in dorsal aorta indicates that this substance has a stimulatory but weak action on the branchial blood vessels

In teleosts the perfused branchial blood vessels showed weak constriction in response to histamine whereas systemic blood vessels either showed no response or responded by only a slightly increased resistance. These observations indicate that the decrease in dorsal aortic blood pressure as obtained in the cabezon also reflects a constriction of branchial blood vessels combined with secondary effects on the heart especially since the effect was more marked after intravenous administration than after injection into dorsal aorta The ability of propranolol and the inability of pyrilamine to depress the dilatatory action of large doses of histamine on the perfused branchial blood vessels of the eel shows that this is probably an unspecific effect corresponding to the dilatatory action of histamine on the branchial vascular system in hagfish. If the low tissue levels are considered the histamine dose required to evoke any responses in teleosts seems to be far higher than would be expected if histamine had any significance in relation to the physiological control of vascular smooth muscle but the blood vessels of local vascular beds may respond differently These considerations may of course apply as well to cartilaginous fish and bichir which all lacked vascular manifestations of any response to histamine, and as far as specific responses to histamine are concerned also to hagfish

Insignificant effects of high doses of histamine have previously been reported from a study in the skate (MacKay 1931) whereas Krawkow (1913) found weak vasoconstriction in response to histamine in the perfused gills of the pike Östlund and Fange (1962) perfused the gills from several species of teleosts and observed no vasoconstrictor effect of histamine The present results are in fairly good agreement with these reports However Mott (1951) reported that histamine caused a fall in ventral aortic blood pressure in eel of the same species as that used in the present study in which the only observed response after small doses was branchial vasoconstriction The decrease in blood pressure reported by Mott (1951) occurred concomitantly with a slowing of the heart and a decrease in cardiac output may equally well explain the decreased blood pressure in ventral aorta as may vasodilatation On the other hand she may have been dealing with a similar dilatatory branchial blood vessel response to histamine which was occasionally observed by Östlund and Fange (1962) after administration of large doses of histamine in perfused gill preparations from the viviparous blenny The present study demonstrates this response pattern both in hagfish and in perfused branchial blood vessels from the eel (Fig 2 and 7) and also justifies the reason for considering it as an unspecific histamine effect mediated through stimulation of β adrenergic receptor mechanisms

With respect to actions of 5 hydroxytryptamine in teleosts the perfusion experiments revealed a powerful constriction of branchial blood vessels and thus confirmed and extended the results from a previous study (Östlund and Fange 1962) The fact that both intravenous and intra arterial injections of 5 hydroxytryptamine elicited a depressor response in dorsal aorta which developed more slowly than the corresponding pressor response in ventral aorta indicates that after injection into dorsal aorta the pressure drop did not ensue until the amine had passed the systemic blood vessels without effect and was reaching the branchial blood vessels Seen in

relation to the slightly increased resistance in perfused systemic blood vessels all these observations support the conclusion that blood pressure changes evoked by 5 hydroxytryptamine in teleosts are mainly caused by increased branchial resistance combined with direct or indirect actions on the heart

The manifestations of the stimulatory actions of histamine on the branchial blood vessels in teleosts and of both histamine and 5 hydroxytryptamine on systemic blood vessels in all studied species except lungfish were too inconsistent and trifling to allow evaluation of specificity by addition of blocking agents to the perfusion fluid With regard to the low levels of both amines which were found in most tissues other than those from lungfish the results confirm previous observations (Erspamer 1954 Reite 1965)

In lungfish the results obtained in intact specimens and in perfused preparations reflect strong and specific stimulatory actions of histamine and 5 hydroxytryptamine A comparison of the effects of acetylcholine and biogenic amines on systemic branchial and pulmonary blood vessels in lungfish shows that with respect to histamine and 5-hydroxytryptamine the responses of all vascular beds are qualitatively similar (Johansen and Reite 1968) The rapid decline of the dorsal aortic blood pressure increase produced by histamine may be due to secondary indirect effects

Compared to the dose necessary to elicit vascular responses the histamine content was high in most of the studied tissues from lungfish The effects of compound 48/80 polymyxin B and protamine were blocked by pyrilamine and chlorpheniramine which indicates that their stimulatory action on lungfish blood vessels was probably mediated by released endogenous histamine These three agents are effective histamine releasers in mammals (Paton 1951 Feldberg and Talesnik 1953 Parratt and West 1957 Smith 1958) where their main targets are the tissue mast cells which contain the bulk of the histamine in the mammalian body (Riley and West 1953 Riley 1959) Fish have also numerous mast cells (Jordan 1926 Michels 1938 Arvy 1957) and there is evidence for mast cell degranulation by compound 48/80 in some species (Veil 1956 1957) but judging from the present results no vasoactive substances are released by this agent except in lungfish The observation that depletion of tissue histamine stores in lungfish was achieved with the same physical and chemical agents which destroyed mast cells points to the mast cell as a likely storage site However since the true tissue mast cells were degranulated in tissues treated with o-phthalaldehyde and cells resembling blood basophils were the only ones which showed yellow fluorescence the results are inconclusive

Previous studies on the pharmacology of extravascular smooth muscles mostly performed in intestinal preparations have revealed either no or only weak stimulatory actions of histamine and 5 hydroxytryptamine in jawless vertebrates and cartilaginous fish (Dreyer 1946 Euler and Östlund 1956—57 Valette and Augereau 1958) In teleosts large species variations in the sensitivity of similar preparations are evident the responses varying from no action to marked stimulation following administration of histamine and from slight to powerful stimulatory action after 5 hydroxytryptamine (Bernheim 1934 Dreyer 1946 Euler and Östlund 1956—57

Burnstock 1958, Valette and Augereau 1958) The pulmonary smooth muscles in lungfish are stimulated by both histamine and 5 hydroxytryptamine (Johansen and Reite 1967) As far as effects of other drugs are concerned, reports reviewed by Fänge (1962) show that acetylcholine usually stimulates extravascular smooth muscle preparations from fish, while the action of adrenaline and noradrenaline may be either stimulatory or inhibitory Many of the early studies were performed without appropriate blocking agents Some of the reported actions of acetylcholine and biogenic amines on fish smooth muscle may therefore represent unspecific responses but it should be emphasized that wherever a response to histamine and 5 hydroxytryptamine has been described it has been stimulatory The stimulatory nature of all responses presently obtained in vascular smooth muscle further strengthens the impression that the actions of histamine and 5 hydroxytryptamine on the various smooth muscles of fish are undifferentiated The inhibitory actions of histamine and 5-hydroxytryptamine on mammalian smooth muscles, especially on those of the vascular system thus seem to be phylogenetically rather young compared to the stimulatory actions This appears to be in contrast with the situation for adrenaline and noradrenaline, where the perfusion experiments show that both their inhibitory and their stimulatory vascular actions are fully developed even in jawless vertebrates In elasmobranchs and teleosts, observations on the vascular actions of adrenaline noradrenaline and acetylcholine, which are in general accordance with those made during the present investigation, have been described previously (Krawkow 1913 MacKay 1931, Keys and Bateman 1932 Lutz and Wyman 1932 Östlund and Fänge 1962)

The present investigation provides evidence that the capability of adrenergic receptors to discriminate between the different biogenic amines develops gradually during the evolution of vertebrates Unspecific effects of histamine and 5 hydroxytryptamine are present in hagfish and are probably produced by triggering of adrenergic receptor mechanisms This mode of action is eventually lost The appearance of specific actions of histamine and 5 hydroxytryptamine on vascular smooth muscle occurs in the higher forms of fish All actions observed in fish are stimulatory The actions of 5-hydroxytryptamine become manifest as a powerful constrictor response in the branchial blood vessels of teleosts In lungfish marked vasoconstrictor responses to both histamine and 5 hydroxytryptamine are present in all studied sections of the vascular system

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From the Institute of Physiology, University of Oslo Norway

Ventilation Hypoxia and Pulmonary Vascular Resistance Effects of Changes in Plasma Potassium Concentration

By

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Abstract

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The effects of stepwise rises in plasma potassium concentration on the pressor responses to short (less than 3 min periods of ventilation hypoxia and to vasoconstrictor agents were investigated in isolated blood perfused rat lungs Both hypoxia responses and drug responses were potentiated Plasma potassium ion concentrations above 17 0 to 18 8 meq/l caused dose dependent sustained elevations of pulmonary vascular resistance (PVR) Ventilation hypoxia superimposed on such high PVR did still cause brisk pressor responses Potassium induced vasoconstriction were obtained also in periods with no pressor responses to ventilation hypoxia i e by cooling of the perfusate or late in a perfusion The results suggest that a reduction of the vascular smooth muscle cell ratio $[K^+]_i/[K^+]_o$ by hypoxia induced transcellular leakage of potassium ions is not the sole mechanism for the pressor response to acute ventilation hypoxia.

It is well known that acute ventilation hypoxia elicits pulmonary vasoconstriction. There is however no general agreement as to the nature of the intrinsic mechanism causing this response *Bergofsky and Holtzman (1967)* have reported that a reversible loss of potassium is seen from specimens of pulmonary artery smooth muscle incubated in hypoxic (3 % O_2) Ringer s solution Such a hypoxia-induced loss was not observed from the smooth muscles of other types of vessels These authors suggest that depolarization of the pulmonary smooth muscle by such changes in the electrolyte concentration which brings the muscle closer to its excitatory threshold might explain wholly or in part the pulmonary vasoconstrictor response to hypoxia

In the present study on isolated perfused rat lungs increases in plasma $[K^+]$ were induced by addition of isotonic potassium salt solutions The qualitative effects of these increases on pulmonary vascular resistance (PVR) and on the pulmonary vascular response to various vasoconstrictor stimuli including acute ventilation hypoxia, were observed It was hoped that such knowledge would be helpful in further elucidating the mechanism for this peculiar effect of hypoxia.

Methods

Isolated rat lungs were perf
positive pressure inflat
recorded Cooling and
changing the water in t
chamber Control tempe
fusate volume varied fro

Acute ventilation hypoxia was created by changing ventilation gas from a 21 % O_2 gas mixture to a 2 % O_2 gas mixture for a period of 30 sec to 3 min (constant in each test ... was N_2 The ... r responses to ... the prepara

Vasoactive drugs were injected into the pulmonary artery tubing in volumes of 0 1 to 0 5 ml For control was used 0 5 ml isotonic NaCl solution (0 9 g/100 ml) Isotonic solutions of KCl (1 12 g/100 ml) and KNO_3 (1 53 g/100 ml) were added to the open blood reservoir

For electrolyte analysis (K^+, Na^+) blood samples (0 3 to 0 5 ml) were taken in small test tubes from the venous outflow tubing and measured in an Eppendorf flame photometer Double determinations were made of the potassium concentration in each plasma sample and the mean value was used Samples were taken during situations with stable PVR, usually just before a test with ventilation hypoxia

The following drugs were used Adenosine 5 triphosphate (ATP, International Chemical and Nuclear Corporation) bradykinin (Synthetic Bradykinin, BRS 640, Sandoz A G), kallidin (Synthetic Kallidin KL 698 Sandoz A G), noradrenaline (l noradrenaline d bitartrate monohydrate Philips Roxane)

Results

Isotonic solutions of either KCl or KNO_3 were added to the blood reservoir in volumes of 0 2 to 0 5 ml By successive such additions plasma [K^+] could be elevated in a stepwise manner In 8 perfusion expts (4 with each potassium salt) such additions invariably increased the magnitude of the pressor responses to ventilation hypoxia, as demonstrated in Fig 1 The effect was dose dependent. KCl and KNO_3 appeared to have similar potency when compared on a molar basis The addition of potassium salt solutions did not in itself cause elevation of pulmonary vascular resistance (PVR) until a plasma concentration of 17 0 to 18 8 meq/l was reached Increasing the plasma [K] from this level did however cause increasingly higher PVR levels

Fig 2 demonstrates two pressor responses to ventilation hypoxia obtained at normal plasma [K] Thereafter 1 ml of isotonic KNO_3 was added to the blood reservoir increasing plasma [K] to 21 8 meq/l This addition caused a doubling of PVR as indicated by a rise in pulmonary artery pressure A test of ventilation hypoxia superimposed at this high level of PVR elicited a pressor response with an amplitude even greater than that of the previous responses Similar results ... tained in three additional experiments

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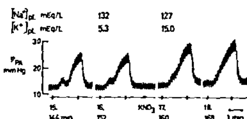


Fig 1

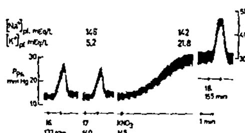


Fig 2

Fig 1 Press obtained before and after the addition of 0 5 ml isc

Isolated p the hypoxic test and time after start of perfusion ar poxia P_{PA} pulmonary artery pressure pl plasma At KNO_3 addition of salt solution Vertical bars mark beginning and end of hypoxic periods

Fig 2 Pressor responses to acute ventilation hypoxia obtained before and after the addition of 1 ml of isotonic (1 53 g/100 ml) KNO_3 solution

Isolated perfused rat lung preparation Number of hypoxic test and time after start of *perfusion are given for each period of ventilation hypoxia* P_{PA} pulmonary artery pressure pl plasma At KNO_3 salt solution added Vertical bars mark beginning and end of hypoxic periods

longer time Addition of potassium salt solutions to the perfusate in this late period elevations of PVR similar to those seen in periods with brisk pressor responses hypoxia (2 expts)

Acute reduction of the perfusate temperature to 27° C abolishes the response to ventilation hypoxia (Haavik Nilsen and Hauge 1968) Elevation of plasma [K] above 18 5 meq/l did however, cause rise in PVR at 27° C (2 expts)

Addition of potassium salt solution to the perfusate potentiated the vasoconstrictor effects of bradykinin kallidin, noradrenaline and to a lesser degree, ATP An example of this is given in Fig 3 where it is demonstrated that an elevation of plasma [K] to 13 meq/l had a larger effect on the response to kallidin than it had on that to acute ventilation hypoxia Equimolar concentration of isotonic NaCl solution had no apparent effect on the response to either stimuli

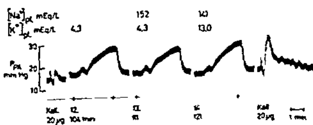


Fig 3 Pressor responses to kallidin (Kall) and to acute ventilation hypoxia obtained before and after the addition of 1 ml isotonic KCl (1 12 g 100 ml)

Isolated perfused rat lung preparation Number of hypoxic test and time after start of perfusion are given for each period of ventilation hypoxia KCl-solution was added between test no 13 and 14 Kallidin was injected 60 and 127 min after start respectively P_{PA} pulmonary artery pressure pl plasma Vertical bars mark beginning and end of hypoxic periods

Discussion

The electric and contractile activity of vascular smooth muscle is partly determined by the ratio $[K^+]/[K^+]_o$ (Bohr *et al* 1958 Axelsson *et al* 1967) In the present tests this ratio was reduced in isolated rat lungs by elevation of extracellular (plasma) $[K^+]$, assuming a constant or almost constant intracellular potassium concentration in the test periods (Dodd and Daniel 1960) Such a maneuvre increased the vasoconstrictor responses to various stimuli including acute ventilation hypoxia and it could also by itself cause elevations of PVR Two different potassium salts were used in order to ascertain that the observed effects were not caused by the anions The decrease in sodium concentration following potassium salt additions is in agreement with the observation by Kjellmer (1965) that potassium infusion into the vascular bed of cat calf muscles induced a fall in effluent blood sodium concentration The changes in $[Na^+]$ are probably much too small to affect vascular tone (Friedman and Friedman 1963)

Since the pressor response to a standardized period of ventilation hypoxia increases and decreases with time in this preparation (Hauge 1968a) a determination of the quantitative relationship between plasma $[K^+]$ and the hypoxic response was not attempted

If a reduction of the transcellular potassium concentration gradient and a resultant reduction in the smooth muscle membrane potential caused by a transcellular leakage of potassium is the mechanism for hypoxia induced pulmonary vasoconstriction one might expect that vasoconstriction due to lowering of the potassium gradient by elevation of $[K^+]_o$ would reduce the pressor response to ventilation hypoxia. It was found however that a KCl or KNO_3 induced sustained elevation of PVR to well above the level seen as a response to hypoxia had no such inhibitory action Also vasoconstriction by potassium salt additions could be obtained in periods where no pressor responses to acute alveolar hypoxia could be elicited [illegible] by cooling of the perfusate to 27° C or late in a perfusion

Bergofsky and Holtzman (1967) using isolated plasma perfused cat lungs found that an elevation of perfusate $[K^+]$ to 9 5 meq/l induced a rise in PVR In the present preparation no rise in PVR was seen until approximately twice that concentration was reached In spite of this difference in responsiveness to perfusate $[K^+]$ elevations the rat lung preparation appeared to respond more vigorously to ventilation hypoxia at normal plasma $[K^+]$ than did the isolated cat lungs In both preparations elevation of perfusate $[K^+]$ potentiated the vasoconstrictor response to hypoxia

A reduction of the transcellular potassium concentration gradient due to an elevation of plasma $[K^+]$ is unphysiological and not directly comparable to a similar reduction caused by fall in intracellular $[K^+]$ Nevertheless the present results suggest that reduction of the $[K^+]/[K^+]_o$ ratio in smooth muscle cells of pulmonary vessels caused by transcellular leakage of potassium is [illegible] the sole mechanism [illegible] hind the vasoconstrictor effect of acute alveolar hypoxia [illegible]

[illegible] al ir trapu [illegible]

changes are apparently involved Some observations indicate that intrapulmonary liberation or formation of a humoral factor (Lloyd 1968), possibly histamine (*Hauge 1968 b Hauge and Melmon 1968*), *takes place during hypoxia The vaso* constrictor effect of such a substance could well be potentiated by transcellular leakage of potassium ions the latter change thus contributing to the very marked pressor responses to hypoxia

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Vago-Vagal Reflex Relaxation of the Stomach in the Cat

By

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Abstract

JANSSON, G, *Vago-vagal reflex relaxation of the stomach in the cat.* Acta physiol scand 1969 75 245—252

In acute experiments on chloralosed cats gastric motility was recorded with a rubber balloon system kept at constant intragastric pressure under experimental conditions not requiring abdominal surgery.—Electric stimulation of the central end of either a small vagal branch at the level of the diaphragm or of one vagal nerve at the level of the neck, while the opposite vagal nerve was left intact produced profound reflex relaxation of the stomach Also the efferent pathway of the relaxatory reflex was in the vagal nerves—The reflex relaxation persisted after administration of atropine, which shows that it was not due to reflex suppression of prevailing activity of the vagal cholinergic motor fibres It was also independent of adrenergic mechanisms, since it persisted after administration of guanethidine in doses known effectively to block the adrenergic intestino gastric inhibitory reflex This vago-vagal relaxatory reflex response thus showed the same characteristics as the gastric response elicited by efferent stimulation of the high-threshold vagal relaxatory fibres—It is concluded that this vago-vagal reflex originates, at least partly, from abdominal structures, with the efferent pathway consisting of the relaxatory, high threshold fibres producing pronounced gastric relaxation This vago vagal relaxatory reflex may be involved in receptive relaxation of the stomach

Besides the well-known excitatory motor fibres the efferent vagal supply to the stomach contains high-threshold efferent fibres that produce marked relaxation of the corpus-fundus region (Jansson and Martinson 1965) The relaxatory response is not blocked by guanethidine or by atropine (Martinson 1965 a) It was suggested that these "relaxatory" fibres might be involved in "receptive relaxation" of the stomach

However, as yet little is known about the mechanisms involved in receptive relaxation This phenomenon appears to be a phasic response associated with swallowing and adapts the volume of the stomach to the amount of food swallowed (Cannon and Lieb 1911—12, Lind *et al* 1961) suggesting that receptors engaged in the elicitation of its reflex are situated in the esophagus or in the stomach Mucosal chemoreceptors and mechanoreceptors have been described in the gastric and intestinal wall as well as in the esophagus (for ref see Paintal 1963), and most of these receptors appear to be connected to unmedullated afferent fibres in the vagal nerves (Iggo 1958)

Electric stimulation of afferent vagal nerves elicits different sorts of motor responses of the stomach (for ref see Harper *et al* 1959) It has for example, been observed

that afferent vagal stimulation can produce both gastric excitation and inhibition (Harper *et al* 1959, Cragg and Evans 1960) However, no attempts were made in these studies to identify the efferent fibres and nervous mechanisms involved In addition, intragastric pressure recordings were utilized, which are hardly suitable for recordings of relaxatory responses of the corpus-fundus sections of the stomach The aim of the present study was to find out with a method recording gastric volume whether "relaxatory" vagal fibres are involved in vago-vagal reflexes

Material and methods

24 cats weighing between 2 4 and 5 5 kg were used The animals were deprived of food for 24—36 hrs before the experiment The anesthesia was induced with ether, and chloralose (40—80 mg/kg) was given i v A free airway was secured by means of a tracheal cannula In the beginning of the experiment the stomach was emptied by suction through a polyvinyl catheter introduced via the esophagus

Recording of gastric volume changes with a rubber balloon Gastric volume was recorded at constant intraluminal pressure by means of a fluid filled system. To avoid the traumatic effect of laparotomy which often *per se* leads to profound gastric relaxation a rubber ballon connected to a polyvinyl large bore catheter was passed down into the stomach via the esophagus The rubber balloon was 10 to 13 cm in diameter and thus large enough to flatten out and fill the cat's stomach without distention of the wall of the balloon Before introduction into the stomach the balloon was completely emptied by suction through the polyvinyl catheter, 5—6 cm of which was situated within the balloon The volume of the deflated balloon together with that part of the catheter in the balloon was about 16—20 ml which was included in the volumes mentioned in the subsequent figures The other end of the catheter was connected to a volume reservoir and the system was filled with water kept at 38 C The volume reservoir was in turn fitted to a float recorder writing on smoked paper

In most experiments the intragastric pressure was kept constant at about 3—6 cm throughout experiment. The level of the pubic tubercle of the cat placed supine on a horizontal table taken as the zero reference for the measurement of intragastric pressure In 6 expts imal vago-vagal relaxatory reflex responses were studied at different intragastric pressures ranging from 1 to 15 cm H_2O

After each experiment an incision was made in the abdominal and stomach wall to check that the balloon was in proper position In addition to the rubber balloon the stomach contained a small volume of gastric juice

Afferent vagal fibres were stimulated at the cervical level or at the diaphragmatic level In the former case the central end of the main vagal trunk on either side was electrically stimulated while the opposite vagal nerve was left untouched In the latter case the abdominal vagal trunks surrounding the esophagus at the level where it passes through the diaphragm were approached by a thoracic incision between the 9th and 10th rib For afferent stimulation either the main trunk dorsally to the esophagus or one of the minor branches was cut just above the diaphragm. The central end was placed in an annular bipolar silver electrode and connected to a Grass Stimulator Model S4 CR Afferent stimulations of 4—30 imp/sec at 5—12 V and a pulse duration of 1 3 msec were used For efferent vagal stimulation 1 20 imp/sec was used at 5—10 V and a pulse duration of 0 05 2 msec

In 5 expts. the spinal cord was completely transected between vertebrae C6 and C7 In two of these experiments spinal anesthesia at the lumbar and thoracic levels was induced by subdural administration of 2 ml of 2 % lidocaine solution Xylocain® Astra through a fine polyethylene catheter

Arterial pressure was measured in a femoral artery and artificial respiration was given continuously in most of the experiments since skeletal muscle activity was blocked by gallamine triethiodide (Flaxedil® May & Baker to avoid disturbances of the gastric responses Atropine was given in a dose of 0 2 1 mg/kg b w (Atropine sulphate Merck and guanethidine (Ismelin® Ciba i v in a dose of 3 5 mg/kg b w

Results

Effects of efferent vagal stimulation on gastric motility recorded with a balloon-volume method" On electric stimulation of the peripheral ends of the cut cervical vagal nerves characteristic stomach responses were recorded with the balloon-

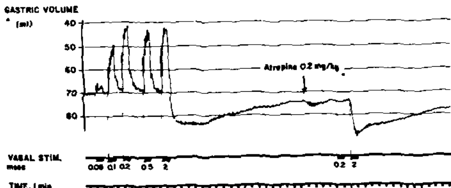


Fig 1 Cat 3 9 kg Gastric volume changes elicited by electric stimulation of the *peripheral* ends of the sectioned cervical vagal nerves at constant frequency (2 imp/sec) and voltage (5 V) but with increasing duration of stimulations Selective excitatory gastric motor responses were recorded at stimulations of 0 05—0 5 msec On stimulation at 2 msec there was still a pronounced excitatory motor response, but now there was a poststimulatory relaxation After atropine (0 2 mg/kg) the low threshold vagal excitatory fibres were blocked revealing the effect of the high threshold *vagal efferent fibres in the form of an immediate long lasting re* laxation

volume method When the balloon system was kept at constant intraluminal pressure stimulation of suitable strength elicited the characteristic relaxatory *response of the* stomach (Jansson and Martinson 1965) Fig 1 illustrates such an experiment where the duration of the impulses was increased step-wise, while the frequency and voltage were kept constant at 2 imp/sec and 5 V, respectively As shown earlier, with this procedure it is possible selectively to stimulate the vagal *efferent excitatory fibres* with stimulations of short duration Pulses of longer duration still elicited an excitatory motor response, but then the stimulation was followed by a characteristic long lasting relaxation Atropine completely blocked the excitatory motor responses and then the high threshold stimulation produced *immediate vagal relaxation* (see also Jansson and Martinson 1965) Efferent stimulation of the cut vagal nerves was regularly done at the end of the experiment to test the efficiency of the recording system in the respective experiments as well as to enable comparison of the appearance of these responses with those of the vago vagal reflex

Vago-vagal reflex relaxation of the stomach Electric stimulation of the central end of a cut vagal nerve produced clearcut pronounced relaxation of the stomach within 5—7 sec provided other vagal branches to the stomach had been left intact It was possible to elicit these reflex relaxatory *responses no matter which of the vagal* trunks was stimulated at the cervical level and whether the right or left vagal nerve was intact (Fig 2A and 4) From Fig 3 it is obvious that afferent stimulation of a small vagal branch at the level of the diaphragm also elicited a reflex relaxation suggesting that at least some of the afferent fibres *emanate from abdominal structures*

Fig 2 Cat 3 0 kg Effect of the vago-vagal relaxatory reflex on the stomach in an atropinized cat (0 2 mg/kg b w)
A The divided right vagal trunk was stimulated at the cervical level in the afferent direction while the left vagal trunk was intact
B The vago-vagal relaxatory reflex remained intact after spinal cord transection between vertebrae C6 and C7
C After bilateral vagotomy the reflex relaxation disappeared Note however that stimulation of the peripheral part of the sectioned left vagal nerve elicited a longlasting relaxation similar to that recorded as a vago-vagal reflex response in A and B Vagal afferent and efferent stimulations at 10 imp/sec 2 msec 8 V

The magnitude of the reflex gastric relaxations was often very pronounced In the experiments in Fig 2 and 3 there was an increase of about 20—30 ml in the volume of the stomach on vagal afferent stimulation Fig 4 illustrates three consecutive tracings of maximal activation of the vago-vagal relaxatory reflex measured at increasing intragastric pressures after atropinization and transection of the spinal cord The vago-vagal relaxatory reflex then increased the volume by up to 100 ml implying that the prestimulatory volume could be more than doubled

The reflexly elicited relaxations of the stomach were transmitted both in afferent and efferent direction by the vagal nerves thus constituting a vago-vagal reflex. When stimulation was performed at cervical level this was confirmed by sectioning the opposite vagus nerve which eliminated the reflex relaxatory response (see Fig 2C) Further the gastric relaxations elicited by vagal afferent stimulation at the diaphragmatic level disappeared after section of the other vagal nerves at this level or after bilateral vagotomy at cervical level On the other hand reflex relaxations persisted after transection of the spinal cord (Fig 2 and 4) after spinal anesthesia and after guanethidine blockade of the adrenergic supply to the stomach Vago-vagal reflex relaxations also persisted after atropine blockade of the vagal excitatory

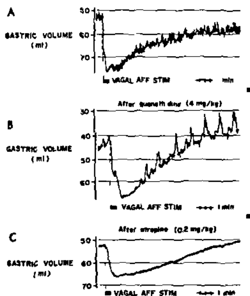


Fig 3 Cat 4 1 kg Gastric reflex relaxatory response upon afferent electric stimulation of a small vagal branch just cranial to the diaphragm

A Vagal afferent stimulation before any drugs had been given

B The vagal stimulation was repeated 20 min after i v administration of guanethidine (4 mg/kg)

C The relaxatory response on vagal afferent stimulation persisted after atropine 0 2 mg/kg b w given i v Vagal afferent stimulation at 10 imp/sec 3 msec, 9 V Artificial respiration and repeated doses of Flaxedil® were given during the experiment

cholinergic fibres to the stomach suggesting that the nervous impulses eliciting the relaxations were mediated by the high threshold vagal efferent relaxatory fibres first described by Martinson (1965 a) Further evidence of this was the similarity in appearance of the reflex relaxatory response (Fig 2 and 4) and of the stomach elicited upon efferent stimulation of the relaxatory vagal fibres (Fig 1 and 2) From the experiments in the atropinized animals (Fig 2 and 4) it is clear that the reflex relaxatory responses were of long duration and that the prestimulatory volume measured at intragastric pressures of 4—6 cm H_2O was not regained until 15—20 after the end of stimulation

It was also observed that abdominal surgery appeared to cause a marked activation of this vago vagal relaxatory reflex with subsequent profund widening of the stomach For such reasons it was often impossible to produce any significant additional vago-vagal reflex relaxation of the stomach after extensive abdominal surgery since the reflex was often maximally or nearly maximally activated

Discussion

The present observations of the reflex control of stomach motility show that stimulation of afferent vagal fibres emanating at least partly from the abdomen can produce profound gastric relaxation This relaxation was shown to be refractory to

Fig. 4 Cat 3.4 kg The effect of the vago-vagal relaxatory reflex on the cat's stomach recorded consecutively at three different intragastric pressures—3.5, 10 cm H_2O—*after administration* of atropine (0.5 mg/kg) and transection of the spinal cord The cut right vagal nerve was stimulated at the cervical level for one minute in the afferent direction with 20 imp/sec, 3 msec and 10 V while the opposite vagal nerve was left intact Spinal cord transection had been performed between vertebrae C6 and C7 two hours before the first recording

cholinergic and adrenergic blocking agents and the efferent pathways were found to run in the vagal nerves The efferent pathway of the described vago-vagal reflex therefore seems to be identical with the high-threshold vagal fibres (Martinson 1965 b), which exhibit exactly the same characteristics with respect to type of response and sensitivity to drugs The reflex utilizing these efferent fibres may therefore be called the vago-vagal relaxatory reflex since both the afferent and the efferent pathways run in the vagal nerves and since the response consists of a pronounced relaxation of the corpus-fundus region of the stomach

In earlier studies on vago-vagal reflexes affecting stomach motility both augmentatory and inhibitory responses have been reported by various authors (for ref see Harper *et al* 1959) These investigators observed that inhibitory long lasting responses could be elicited in most experiments Cragg and Evans (1960) also recorded a reduction in gastric tone on afferent vagal stimulation But in neither case was any attempt made to identify the efferent pathways or the type of transmission involved

In the gastric and intestinal walls stretch receptors and mucosal chemo and mechanoreceptors have been described (for ref see Paintal 1963) Most of these receptors seem to be connected to unmedullated afferent fibres running in the vagal nerves (Iggo 1958) Iggo (1955) showed that the spike discharge from mechanoreceptors in single afferent vagal fibres varies linearly with the pressure in the distended stomach Agostoni *et al* (1957) found that the vagal nerve content of afferent fibres was about 90 % of the 30 000 vagal fibres present at the level of the diaphragm In the present investigation a vago-vagal reflex relaxation of the stomach was recorded on electric stimulation of afferent vagal fibres, at least some of which emanate from the abdominal cavity However, the type of receptors capable of initiating such relaxatory responses is not yet known, but it seems likely that some of the above mentioned mechano- and chemoreceptors of the gastro-intestinal tract possess this ability

The present results were obtained with the aid of a gastric recording system measuring volume instead of pressure Earlier investigations have been performed with some kind of pressure recording system However, volume and pressure recording systems measure different functions of the stomach (Jansson and Martinson 1965, Martinson 1965 a, b) The relaxation of the stomach described here is only poorly revealed by a pressure recording system (Martinson and Muren 1963), while its effect on gastric volume is profound and readily demonstrated

Another methodological problem is that experimental procedures involving abdominal surgery easily elicit a background discharge in afferent fibres with reflex connections to different autonomic fibres with the consequence that it may be impossible to record any addition of a reflex relaxatory response on afferent vagal stimulation By recording gastric motility at low constant intragastric pressure with a balloon inserted *via* the esophagus it was possible to avoid abdominal surgery and thereby to minimize the background reflex discharge of the vagal relaxatory fibres

Vagally induced relaxation of the stomach can be accomplished in two ways 1) By inhibition of pre existing activity of the vagal excitatory fibres to the stomach 2) By suppression of the myogenic tone of the stomach by excitation of the relaxatory fibres In the present experiments atropine was used to block the excitatory cholinergic effects on the stomach The vago-vagal relaxation remained also after administration of atropine which argues against the possibility of an inhibition of pre existing nervous discharge in vagal excitatory cholinergic nerves being responsible for the vago-vagal relaxatory reflex This does not preclude involvement of such a mechanism as cause of a part of the reflex relaxation on vagal afferent stimulation In the present experiments as well as in experiments performed by Babkin and Kite (1950) some observations suggested participation of a central inhibition of vagal excitatory tone For instance the relaxatory response before atropine was often greater than after atropine which might perhaps be ascribed to a reflex inhibition of prevailing activity in vagal excitatory fibres

It should be observed that also adrenergic inhibitory reflexes act upon the gastric neuro-muscular system Thus distention of *e g* an intestinal loop produces an in

testino-gastric inhibitory reflex, which is a reflex dependent on spinal connections and utilizing an adrenergic peripheral mechanism (Jansson and Martinson 1966) But this latter reflex operates mainly by inhibiting existing cholinergic excitatory motility, which is not the case with the vago-vagal relaxatory reflex. Further, this latter type of reflex is unaffected by guanethidine, which effectively blocks the intestino-gastric inhibitory reflex.

The stomach needs a receptive relaxation to fulfil its reservoir function and therefore requires a reflex which can elicit profound gastric relaxation However, further information concerning location and type of the receptor which elicits the vago-vagal relaxatory reflex is necessary before it can be proved that this reflex is engaged in normal receptive relaxation of the stomach

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Resting Membrane Potential in Neck and Leg Muscles of Young Rats

By

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Earlier investigations on single muscle fibres of rat and chicken have shown that the muscle membrane potential increases during ontogeny (Fudel Osipova and Martynenko 1962, Boethius and Knutsson 1966) This finding prompted a further interest in membrane characteristics associated with development and initiated the present study on the time relations of membrane potential build up throughout the cephalocaudal axis

The membrane potential was therefore determined in neck muscles and in the gastrocnemius in rats whose ages varied from newborn to 16 days Recordings were made *in situ* with capillary microelectrodes (20—50 mΩ), the reliability of which was checked previously on the frog sartorius Membrane potential was ascertained by driving the microelectrode slowly through the muscle tissue and recording on film sudden negative deflections indicative of membrane penetration Many of the measurements were abnormally low, presumably because of faulty penetrations effected by the randomly-introduced microelectrode To minimize this experimental error only the largest resting potential obtained for each electrode track was used to calculate the mean membrane potential for each age-group The material thus collected consisted of 4—17 measurements per age group

At birth the gastrocnemius membrane potential (solid line in Fig 1) was low and remained at approximately the same level (52—62 mV) during the first days after birth A sizeable increase in the mean membrane potential did not begin until the 4th day After this latent period a phase of rapid increase in potential followed so that by the 10th day the potential approached the value found for adult animals The mean resting potential of the neck muscle fibres (dotted line in Fig 1) was considerably higher at birth than that of the gastrocnemius muscle and increased significantly already during the first two days after which period it approached adult values

The developmental pattern of the gastrocnemius membrane potential in rat characterized by a steep rising phase agrees with that recorded for muscle membranes in chick embryos (Boethius and Knutsson 1966) The diagram demonstrates that there is a cephalo-caudal gradient for the development of the muscle membrane potential It also indicates that during development the increase of the muscle

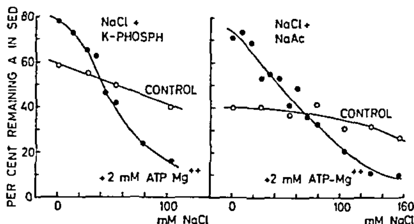


Fig 1 Isolated adrenal medullary granules incubated in K phosphate + NaCl in different proportions and NaAc + NaCl (+80 mM Tris maleic acid) pH 7 5 60 min 37° C No addition (control) (○—○), addition of 2 mM ATP Mg (●—●) Ordinate remaining A in sediment in per cent of original amount Abscissa concentrations of NaCl in medium

acetate Tris buffer ADP acted similarly as ATP, and in both cases Mg^{2+} markedly enhanced the effect

The present experiments have thus shown that the concentration of chloride ions in the medium is an important factor in determining the effect of ATP Mg on the release rate of NA and A from adrenal medullary granules

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The Effects of Hypercapnia and Hypocapnia upon the Cerebrospinal Fluid Lactate and Pyruvate Concentrati and upon the Lactate, Pyruvate, ATP, ADP, Phosphocreat and Creatine Concentrations of Cat Brain Tissue

By

LARS GRANHOLM and BO K. SIESJÖ

Received 14 March 1968

Abstract

GRANHOLM, L and B K SIESJÖ, *The effects of hypercapnia and hypocapnia upo cerebrospinal fluid lactate and pyruvate concentrations and upon the lactate, pyr ATP, ADP, phosphocreatine and creatine concentrations of cat brain tissue.* Acta ph scand 1969 *75* 257—266

The pronounced effects of hyper- and hypocapnia upon cerebral funtion (se Woodbury and Karler 1960) have led to inquiries into the mode of action of ca dioxide upon the brain Some of the effects of carbon dioxide are probably secor to changes in cerebral blood flow (CBF), and it has been claimed that a pronou hyperventilation can decrease CBF to such a degree that cerebral hypoxia e (Kety and Schmidt 1946, Sugioka and Davis 1960, Meyer and Gotoh 1960 Al der *et al* 1965) However, apart from the effects of varying P_{CO_2} levels upon the bral blood flow, there are indications from *in vitro* studies that carbon dioxid more direct effect on cerebral metabolism (Domonkos and Huszak 19 latter effect is evident also *in vivo* if tissue lactate and pyruvate concentrati

measured (Bain and Klein 1949, Leusen and Demeester 1966, Plum and Posner 1967) In general, these results have shown that hypercapnia leads to a decrease, and hyperventilation to an increase, in the tissue concentrations of these acids

There has recently been a renewed interest in the lactate and pyruvate concentrations of tissues and extracellular fluids since it has been pointed out that the lactate/pyruvate ratio reflects the redox state of the cytoplasmatic $NADH/NAD^+$ system (Huckabee 1958, Hohorst 1960, Williamson, Lund and Krebs 1967) In other words, measurements of the lactate/pyruvate ratio of a tissue, and possibly also of an extracellular fluid in diffusion equilibrium with the tissue, may give valuable information on the presence of tissue hypoxia This general assumption has recently been validated for brain tissue since it has been shown that a lowering of the cerebral perfusion pressure is accompanied by an increased cerebrospinal fluid (CSF) lactate/pyruvate ratio (Siesjö, Kjallquist and Zwetnow 1968, *cf* also Zwetnow, Kjällquist and Siesjo 1968) A corresponding analysis of the lactate/pyruvate ratio of cat CSF during passive hyperventilation showed a clear increase in the ratio when the arterial CO_2 tension was reduced below 20—25 mm Hg (Granholm and Siesjö 1968) These results were thus in disagreement with those reported by Leusen and Demeester (1966), who reported a decreased tissue lactate/pyruvate ratio in hyperventilated rats, but, in that study the arterial CO_2 tension was not measured, and the hyperventilation was thus not accurately defined

The evaluation of tissue redox states from the appropriate lactate/pyruvate ratios ures knowledge of the concomitant acid base changes Thus, since hydrogen ns participate in the equilibrium reaction between the $NADH/NAD^+$ and the lactate/pyruvate systems (see Discussion), it must be expected that the lactate/pyruvate ratio increases and decreases in hyper- and hypocapnia, respectively, assuming a constant NAD reduction (*cf* Tobin 1964)

The present experiments were designed to study the effects of hyper- and hypocapnia upon lactate and pyruvate concentrations in CSF and in brain tissue, and upon tissue concentrations of ATP, ADP, phosphocreatine and creatine A special emphasis was laid on pronounced hyperventilation ($PaCO_2 < 20$ mm Hg) In order to evaluate the effects of hydrogen ion changes on the equilibrium between the lactate pyruvate and $NADH/NAD^+$ systems, the mean intracellular bicarbonate concentrations were calculated and the theoretically expected changes in the lactate/pyruvate ratios were compared to the experimental ones It will be shown that the experimental lactate pyruvate ratios in the tissue varied according to the calculated intracellular bicarbonate concentration in hypercapnia During hyperventilation, however, the lactate pyruvate ratios of both the tissue and the CSF increased markedly *above the expected ratios, suggesting the presence of tissue hypoxia*

Methods

The experiments were made on adult cats of both sexes weighing 1 5 to 3 kg The animals were anaesthetized and prepared as described in a preceding paper (Granholm *et al* 1968 with cats in the control group breathing air spontaneously Hypercapnia was studied in the experimental group by administering CO_2 gas mixtures 6 9 or 15 % CO_2 in 30 % O_2 to the spontaneously breathing animals. Hyperventilation was studied by increasing mainly the volume of ventilation

groups

$$K = \frac{(\text{Pyruvate})\,(\text{NADH})(\text{H}^+)}{(\text{Lactate})\,(\text{NAD}^+)} \qquad (1)$$

was determined by Williamson *et al* (1967) to be 1 11 × 10⁻¹¹ mole/l This constant thus makes it possible to calculate the intracellular (cytoplasmatic) NADH/NAD+ ratio provided the intracellular lactate, pyruvate and hydrogen ion concentrations are known. In a homogeneous system

$$(\text{H}^+) = \frac{P_{CO_2}\ S\ K'}{(\text{HCO}_3^-)} \qquad (2)$$

$$\frac{(\text{Lactate})}{(\text{Pyruvate})} = \frac{(\text{NADH})}{(\text{NAD}^+)} \cdot \frac{P_tCO_2}{(\text{HCO}_3^-)_t} \cdot \frac{S\ K'}{K}.$$

Results

Fig 1 illustrates the lactate and pyruvate changes in cisternal CSF In hypercapnia both lactate and pyruvate decreased but, since there was a proportionally larger decrease in pyruvate, the lactate/pyruvate ratio increased progressively. In hypocapnia, there was a corresponding increase in the lactate and pyruvate concentrations which accelerated steeply at CO_2 tensions below about 20 mm Hg At these low CO_2 tensions there was a progressive rise in the lactate/pyruvate ratios

Fig 2 shows the corresponding lactate and pyruvate concentrations in cortical tissue Both lactate and pyruvate changed in a similar manner as in CSF A comparison between the experimental lactate/pyruvate ratios and those calculated from a theoretical equilibrium between the lactate/pyruvate and NADH/NAD+ systems (see Methods) shows a good correspondence in hypercapnia but a clear disagreement

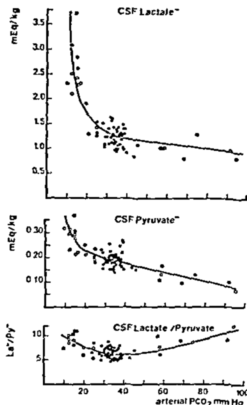


Fig 1

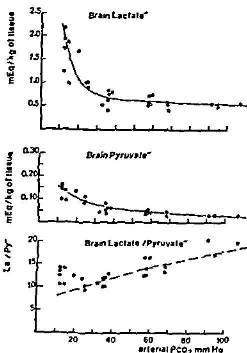


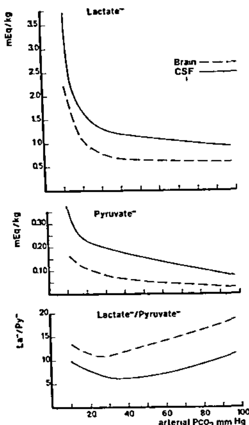
Fig 2

Fig 1 The influence of changes in the arterial CO_2 tension upon the lactate and pyruvate con [illegible]

Fig 2 Lactate and pyruvate concentrations and lactate/pyruvate ratios of cerebral cortical tissue from cats, related to the arterial CO_2 tension. The unbroken lines were drawn by eye to fit the experimental points while the broken line is the theoretical change of the lactate/pyruvate ratio assuming a pH-dependent equilibrium between the lactate/pyruvate and the NADH/NAD$^+$ systems at constant NAD reduction The experimental points coincided with the theoretical curve at high CO_2 tensions but a marked deviation occurred at CO_2 tensions below 25 20 mm Hg

TABLE I Concentrations of lactate pyruvate, ATP, ADP P creatine and creatine in cortical [illegible]ma, P_{aCO_2} in mm Hg hemoglobin (Hb) in g %

	n	P_{aCO_2}	Hb	Lact	Pyr
Hyperventilated animals	9	16 6 ±1 8	13 1 ±0 9	1 516 ±0 152	0 124 ±0 009
Control group	7	34 1 ±1 7	12 4 ±0 5	0 705 ±0 070	0 063 ±0 007
CO_2-breathing animals	10	72 3 ±5 9	11.8 ±0 5	0 572 ±0 031	0 038 ±0 003



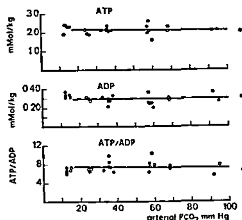


Fig 3 Fig 4

Fig 3 Comparison between lactate and pyruvate concentrations and lactate/pyruvate ratios in cerebral cortical tissue and in cisternal CSF of anaesthetized cats at various CO_2 tensions The lines were drawn by eye as the best fits to the experimental points (see Fig 1 and 2) Note similarity of changes in tissue and CSF

Fig 4 ATP and ADP concentrations and ATP/ADP ratios of cerebral cortical tissue of anaesthetized cats, related to the arterial CO_2 tension (*cf* Fig 1 and 2) The straight lines were drawn by eye as the best fits of the experimental points

tissue from cat brains (in mmoles/kg of wet tissue) in normocapnia, hypocapnia and hypercap-

ATP	ADP	PhCr	Cr	Lact / Pyr	ATP / ADP	PhCr / Cr
2 20 ±0 08	0 32 ±0 01	4 69 ±0.20	6 11 ±0 26	12 1 ±0 6	6 8 ±0 2	0 79 ±0 05
2 13 ±0 04	0.29 ±0 01	4 80 ±0 09	6 02 ±0 27	11 3 ±0 4	7 6 ±0 5	0 80 ±0 03
2 12 ±0 08	0 29 ±0 02	4 32 ±0 17	6 09 ±0 24	15 5 ±0 9	7 5 ±0 4	0 70 ±0 04

tensions increase the cerebral blood flow, hyperventilation can reduce the flow to less than 50 per cent of the basal levels (Kety and Schmidt 1948, Harper and Glass 1965). The variations in flow have their functional counterparts in that marked hyper- and hypocapnia lead to CO_2 narcosis and tendency towards seizures, respectively. However, even small variations in the tissue CO_2 tension have clear effects on the excitability of cortical cells (see review by Woodbury and Karler 1960, Krnjević, Randić and Siesjö 1965), and it is probable that most of the effects elicited by moderately increased or decreased CO_2 levels are mediated by other mechanisms than variations in blood flow. Thus, observations on both brain tissue *in vivo* (Bain and Klein 1949) and *in vitro* (Domonkos and Huszak 1959), as well as on other tissues (Delcher and Shipp 1966, Scheuer and Berry 1967) have indicated that alkalinization of the tissues or its surroundings increase glucose uptake as well as lactate and pyruvate production. These effects have been ascribed to a direct pH effect on the phosphorylation of glucose, and on the phosphofructokinase step in the glycolytic sequence.

Recent studies have confirmed that low CO_2 tensions lead to increases in lactate in both the CSF (van Vaerenbergh, Demeester and Leusen 1965, Plum and Posner 1967) and the brain tissue (Leusen and Demeester 1966). The present results have shown that both lactate and pyruvate in brain as well as in CSF vary continuously and inversely with the arterial CO_2 tension, but that an abrupt increase occurs at CO_2 tensions below 25—20 mm Hg. An even greater importance should be ascribed the variations in the lactate/pyruvate ratio, which was found to agree with the ratio predicted from the pH dependent equilibrium between the lactate/pyruvate and the $NADH/NAD^+$ systems at increased CO_2 tensions, but to deviate from the expected ratio at low CO_2 tensions (see Methods and Results). In view of the assumed coupling between the lactate/pyruvate and the cytoplasmatic $NADH/NAD^+$ systems (Huckabee 1958, Hohorst 1960, Williamson *et al.* 1967, see also Siesjö *et al.* 1968), these results indicate that the $NADH/NAD^+$ ratio increases during pronounced hyperventilation.

The circulatory and functional effects of hyperventilation have led to early assumptions of a hypoxic influence of low CO_2 tensions. These assumptions have gained some qualitative support from EEG studies, and from measurements of tissue oxygen tensions during hyperventilation (Sugioka and Davis 1960, Meyer and Gotoh 1960). Alexander *et al.* (1965) have recently shown that extreme hyperventilation in human volunteers is associated with an increased glucose uptake and an increased lactate release by the brain. However, measurements of the arteriovenous "excess lactate concentrations" or lactate pyruvate ratios have not given conclusive evidence of a tissue hypoxia (Cain 1963, Alexander *et al.* 1965). Moreover, in one study on rats Leusen and Demeester (1966) reported a *decrease* in the brain lactate/pyruvate ratio during hyperventilation, but in that study the arterial CO_2 tension was not measured.

The present results thus seem to give the first unequivocal indication that pronounced hyperventilation is accompanied by tissue hypoxia in the brain. The inter-

pretation of the results is, however, complicated by the findings of unchanged tissue levels of ATP and phosphocreatine That is, if an increased cytoplasmatic $NADH/NAD^+$ ratio is taken to indicate tissue hypoxia, it is difficult to explain the lack of hydrolysis of phosphocreatine and ATP However, since also the mitochondrial $NADH/NAD^+$ system, as studied with the microfluorometric method of Chance *et al* (1962), is reduced during extreme hyperventilation in rats (Granholm, Lukjanova and Siesjo 1968), the redox change seems firmly established Further experiments with different forms of anaesthesia are evidently needed to resolve the discrepancies of the results

It has been shown in other publications from this laboratory that the CSF lactate/pyruvate ratio appears to be a sensitive indicator of tissue hypoxia (Siesjo, Kjallquist and Zwetnow 1968, Kjallquist, Siesjo, and Zwetnow 1968) The present experiments have shown that, although the lactate and pyruvate concentrations and the lactate/pyruvate ratios in CSF and brain tissue differ quantitatively, they change in a very similar manner both in hyper and hypocapnia The combined results thus support the interesting possibility that the CSF lactate/pyruvate ratio closely reflects cellular redox changes A tight coupling between lactate/pyruvate changes in brain tissue and in CSF evidently opens up interesting diagnostic possibilities in clinical medicine

In a previous paper (Granholm *et al* 1968) we were able to demonstrate that the distribution of lactate, but not of pyruvate, approached a theoretical non ionic" distribution according to the pH gradient The present results show a very good agreement between the bicarbonate and the lactate distribution in hypercapnia, and between the bicarbonate and the pyruvate distribution in hypocapnia Although these results indicate that lactate and pyruvate are unequally partitioned between the extra and intracellular spaces, they give no hint towards the mechanisms responsible Future work is needed to decide whether lactate or pyruvate are transported actively by mechanisms sensitive to pH, or whether the distribution of lactate, pyruvate or bicarbonate between the cytoplasm of the cells and other subcompartments is influenced by acid base shifts

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Acta physiol. scand. 1969. 75. 267—275

From the Neurosurgical Research Laboratory, E-blocket, Lasarettet, Lund, and from the Neurosurgical Departments of the Universities of Gothenburg and Lund, Sweden

Effects of Increased Intracranial Pressure on Cerebral Blood Flow and on Cerebral Venous pO_2, pCO_2, pH, Lactate and Pyruvate in Dogs

By

A KJÄLLQUIST, B K SIESJO and N ZWETNOW

Received 9 April 1968

Abstract

KJÄLLQUIST, A, B K SIESJO and N ZWETNOW *Effects of increased intracranial pressure on cerebral blood flow and on cerebral venous pO_2, pCO_2, pH, lactate and pyruvate* Acta physiol scand 1969 75 267—275

The effects of increased cerebrospinal fluid (CSF) pressure on cerebral blood flow and on cerebral venous pO_2, pCO_2, pH, lactate, and pyruvate were studied in dogs by infusing an artificial CSF into the cisterna magna With moderate and rapid increases in the CSF pressure, which did not lower cerebral perfusion pressure below 60—70 mm Hg, the cerebral blood flow was not measurably lowered, although there were sometimes detectable metabolic changes in cerebral venous blood suggestive of a transient reduction of the cerebral blood flow That is, cerebral venous pO_2 and pH decreased slightly, and there were increases in the cerebral venous pCO_2 in the lactate and pyruvate concentrations, and in the lactate/ [illegible] When the pressure was released there was [illegible] metabolic changes in the cerebral venous [illegible] reased, and the cerebral perfusion pressure [illegible] ere marked metabolic changes in cerebral [illegible] the [illegible] was [illegible] CO_2 [illegible] tive [illegible] hyperemia there were no lactate/pyruv[illegible] of anaerobic glucolysis

It is well known that the cerebral perfusion pressure can be decreased to about 40—60 mm Hg by means of decreases in the arterial blood pressure before the cerebral blood flow (CBF) diminishes significantly, *i e* the brain circulation shows autoregulation (see *e g* Forbes and Wolff 1928, Harper 1963, Rapela and Green 1964, Haggendal and Johansson 1965)

This autoregulation is also seen when the cerebral perfusion pressure is decreased by means of experimental increases in the cerebrospinal fluid (CSF) pressure It has also been shown that when the intracranial pressure is suddenly brought back to normal there is often an increase in the cerebral blood flow of varying magnitude

and duration (Noell and Schneider 1948, Haggendal *et al* 1967, see also Zwetnow, Kjällquist and Siesjö 1968)

In a series of experiments we have studied the effect of increased CSF pressures on some parameters related to the acid base and the energy metabolism of the brain. The present paper describes changes in the cerebral venous pO_2, pCO_2, pH, lactate and pyruvate during and after periods of an acutely increased CSF pressure, as related to the concomitant changes in CBF. The experiments showed that even slight increases in the intracranial pressure, which did not lower the cerebral perfusion pressure below the autoregulation range, where often accompanied by small changes in the venous pO_2, pCO_2, pH, lactate and pyruvate, suggestive of a significant, but possibly transient decrease in the CBF. With more marked decreases in the cerebral perfusion pressure, these chemical changes were more pronounced, and when a reactive hyperemia was elicited, the venous pO_2, pCO_2 and pH usually varied in accordance with the blood flow measured with the clearance technique. Changes in the venous lactate and pyruvate concentrations as well as in the lactate/pyruvate ratios, were seen only during the periods of increased CSF pressure, while there were no changes in the lactate and pyruvate concentrations during the phase of reactive hyperemia, suggesting a previous period of tissue hypoxia.

Methods

The present results were obtained on 18 dogs. 14 of which were anaesthetized with pentobarbital (Nembutal, Abbott, 40 mg/kg i.v., with supplementary doses of 3—4 mg at intervals). A[illegible] animals were immobilized with suxamethonium chloride (Celocurin klorid Vitrum) and [illegible]tilated artificially with a Starling type respirator. Three dogs were anaesthetized with 1 % nitrous oxide in oxygen and one with 2 % halothan ('Fluothane" ICI). In the latter cases anaesthesia was initiated with intravenous thiomebumal sodium (Pentothal Abbott) before intubation with a cuffed rubber tube. The body temperatures were measured rectally and were kept as close as possible to 38° C by means of intermittent heating and cooling.

In all animals one femoral artery was cannulated for blood sampling and recording of pressures. A polyvinylene catheter was introduced into the superior sagittal sinus which was exposed through a craniotomy. The size of the catheter permitted free flow of blood during sampling but did not obstruct the sinus. Clotting was prevented by repeated intravenous administration of small doses of heparin.

The cisterna magna was punctured percutaneously with a double barrelled needle which allowed removal of CSF samples, infusion of artificial CSF and recording of the CSF pressure. The CSF pressure was increased by [illegible] the cisterna magna from a pressure rese[illegible] lowering of the pressure was achieved b[illegible] 1967). The artificial cerebrospinal fluid had the following composition NaCl 124 NaHCO₃ 2[illegible] NaH_2PO_4 H_2O 0.5 $CaCl_2$ 2.3 $MgCl_2$ 6 H_2O 1.6 and KCl 3 meq/l respectively. Both the arterial pressure and the CSF pressure were recorded continuously on a Moseley 2-channel DC *recorder*. Mean pressures were obtained by electrical integration. The cerebral perfusion pressure was defined as the difference between the mean arterial pressure and the mean CSF pressure.

Mean cerebral blood flows as well as cerebral cortical flows were calculated from the tissue clearance of xenon[133] which was injected as a saline solution into the left vertebral artery (Lassen *et al* 1963, Haggendal, Nilsson and Norback 1965, Hoedt Rasmussen, Sveinsdottir and Lassen 1966). In this study only grey matter flow is presented. Since the flow method requires a steady state which was not present in the posthypertensive periods the corresponding CBF values given will only be semiquantitative.

Arterial and cerebral venous blood were analyzed for pO_2, pCO_2 and pH by means of micr[illegible] liquid nitrogen for s[illegible] anholm *et al* 196[illegible] the pO_2 and pH [illegible]

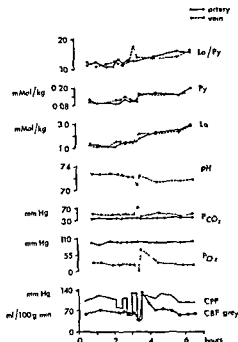


Fig 1 pO_2, pCO_2, pH, lactate and pyruvate in arterial and in cerebral venous blood during three consecutive reductions of the cerebral perfusion pressure, resulting from acute increases in the CSF pressure, that was induced by *infusion of an artificial CSF into cisterna magna* (see text) The cerebral perfusion pressure (CPP) was reduced to 85, 60, and 40 mm Hg, respectively The last reduction of the CPP led to a significant decrease in cerebral blood flow (CBF grey) and was followed [illegible]
ductic [illegible]
decre [illegible]
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site directions while there were no remaining changes in lactate or pyruvate

Results

The present results are based on experiments in 18 dogs In 14 of these experiments, the arterial pCO_2 varied between 30 and 40 mm Hg, while two animals were hypercapnic (pCO_2 about 45 and 65 mm Hg, respectively) and two others were hypocapnic (pCO_2 about 25 and 18 mm Hg, respectively) In all animals except one (pO_2 60—70 mm Hg) the arterial pO_2 exceeded 75 mm Hg The CSF pressure was increased to values between 25 and 105 mm Hg for periods of 3 to 15 min giving reductions of the cerebral perfusion pressure to between 20 and 110 mm Hg In 18 of the 35 pressure periods the reductions in cerebral perfusion pressure were of sufficient magnitude to elicit a subsequent reactive hyperemia In 9 of these 18 runs, CBF was measured also during the period of increased CSF pressure In 7 of the 9 cases there was a clear reduction in CBF In the remaining two cases no such re*duction was apparent, but the CBF measurements were too far apart to exclude a* change in CBF When the CSF pressure was increased but the cerebral perfusion pressure was maintained over 60 mm Hg (8 expts), there was no significant decrease in CBF during the pressure period, and no reactive hyperemia when the pressure was released At cerebral perfusion pressures below about 60 mm Hg, and especially below 40 mm Hg or lower (13 expts), there was usually a marked decrease in CBF during the intracranial hypertension, and subsequently a marked reactive hyperemia

The general layout of the experiments and the scheme of analysis is illustrated in Fig 1, which shows an experiment in which the intracranial pressure was increased

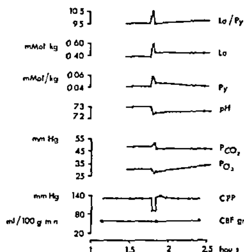


Fig 2 pO_2, pCO_2 pH, lactate and pyruvate in cerebral venous blood during a decrease in the cerebral perfusion pressure (CPP) from 130 to 95 mm Hg (*cf* Fig 1 and text) This moderate decrease in perfusion pressure did not cause any reactive hyperemia but was accompanied by decreases in pO_2 and pH, and of increases in pCO_2 lactate, pyruvate, and in the lactate/pyruvate ratio

to progressively higher values during 3 consecutive runs It is seen that the first two reductions of the cerebral perfusion pressure (to 85 and 60 mm Hg respectively) did not lead to any appreciable changes in either CBF, or in any of the metabolic parameters measured At a cerebral perfusion pressure of 50 mm Hg however, the CBF, as well as the venous pO_2 and the pH decreased markedly, while the venous pCO_2 and the lactate/pyruvate ratio rose When the pressure was released a marked hyperemia ensured during which the pO_2 the pCO_2 and the pH in cerebral venous blood overshot the baselines, and approached the corresponding arterial values

Since cerebral perfusion pressures of 60 mm Hg, or higher, seldom were associated with a decrease in CBF, or with a subsequent reactive hyperemia, and since perfusion pressures between normal values and 60—70 mm Hg are regarded as the autoregulation region (see Discussion), the present material was divided into groups of moderate and marked intracranial hypertensions (cerebral perfusion pressures larger or less than 60 mm Hg respectively)

A Moderate intracranial hypertension (cerebral perfusion pressure higher than 60 mm Hg)

The present results have confirmed that the cerebral perfusion pressure can be reduced to about 60 mm Hg without causing any measurable decrease in cerebral blood flow (Haggendal *et al* 1967) When a normal perfusion pressure was restored after such a decrease there was no reactive hyperemia When cerebral venous blood was sampled during the pressure periods analyses of the pO_2, pCO_2, pH, lactate and pyruvate usually showed very small changes or no changes at all However, in a few experiments there were clear changes in most, or in all of the parameters measured suggesting that the CBF was at least transiently, reduced An example of this is shown in Fig 2 In this experiment the CSF pressure was increased to 35 mm Hg giving a reduction of the perfusion pressure from 130 to 95 mm Hg During the period of increased intracranial pressure the venous pO_2 decreased from 30 5 to

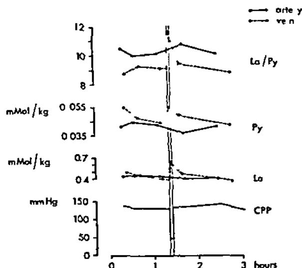


Fig 3 Cerebral venous lactate and pyruvate during a short but pronounced increase in the CSF pressure leading to a reduction of the cerebral perfusion pressure from 130 to 45 mm Hg Note that in spite of a marked increase in the venous lactate/pyruvate ratio during the pressure period there were no remaining lactate/pyruvate changes in blood to suggest that the preceding period of hypoxia had taken place once the pressure had been restored to normal

27 5 mm Hg the pCO increased from 48 3 to 50 4 mm Hg and the pH decreased from 7 29 to 7 24 These changes were accompanied by increases in the venous lactate and pyruvate concentrations (0 18 and 0 015 mmol/kg whole blood respectively), and by an increased lactate/pyruvate ratio (9 5 to 10 3) When such changes were observed all parameters usually returned towards normal values immediately after the pressure was released with no overshoot of the baselines In no case could any significant cerebral venous changes be detected after such a moderate intracranial hypertension had been released

B Pronounced intracranial hypertension (cerebral perfusion pressure less than 60 mm Hg)

Reductions of the cerebral perfusion pressure to values below 60 mm Hg usually gave a measurable reduction of the CBF and a subsequent reactive hyperemia of varying magnitude and duration. When the cerebral perfusion pressure was reduced to 40 mm Hg or lower, these changes invariably resulted Pronounced decreases in cerebral perfusion also led to very marked changes in cerebral venous blood during the phase of reduced flow, and to conspicuous changes during the period of reactive hyperemia as well During the intracranial hypertension cerebral venous pO_2 and pH decreased while cerebral venous pCO lactate pyruvate and the lactate/pyruvate ratios usually increased Immediately after the intracranial hypertension, *i e* during the period of reactive hyperemia cerebral venous pO_2 pCO_2 and pH changed in opposite directions The most marked changes occurred in the venous pO which could decrease to 10 mm Hg during a pressure period and increase towards arterial values during the period of reactive hyperemia

Fig 3 illustrates an experiment in which the cerebral perfusion pressure was reduced to 45 mm Hg for 3 minutes. During the period of increased pressure the lactate and pyruvate concentrations in cerebral venous blood increased by 0 25 and

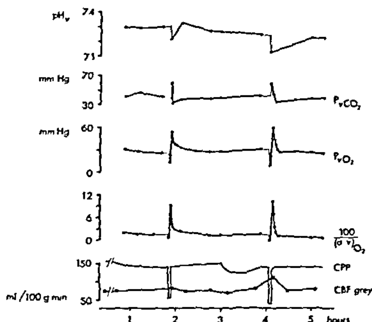


Fig. 4 Cerebral venous pO_2, pCO_2 and pH related to the ... (CPP) and ... in the CSF ... intracranial ... intracranial pressure was restored to normal, pO_2, pCO_2 and pH changed in accordance with a hyperemia. The relative flow calculated from the arterio-venous oxygen differences, on the assumption of a constant oxygen consumption, illustrates the non steady state nature of the clearance values in the early phases of reactive hyperemia

0.01 mmoles/kg respectively and the lactate/pyruvate ratio increased from 9.0 to 11.5 The figure also illustrates the general finding that such lactate and pyruvate changes were quickly normalized after the pressure was released

In Fig 4 changes in venous pO_2, pCO_2 and pH, along with arteriovenous oxyge differences are shown during and after two consecutive reductions of the cerebr perfusion pressure During the first reduction of the cerebral perfusion pressure (t 45 mm Hg), the venous pO_2 decreased from 28 to 16 mm Hg and the pCO_2 in creased from 45 to 63 mm Hg When the pressure was released pO_2 momentanl increased to a peak value of 56 mm Hg, and pCO_2 fell to 37 mm Hg Simila changes were seen during the second pressure period (cerebral perfusion pressur reduced to 30 mm Hg) but in this latter case the pO_2 was reduced to 13 mm H during the hypertension, and increased to a peak value of 63 mm Hg during th reactive hyperemia

In general, reductions of the cerebral perfusion pressure to levels which gave rise to a subsequent reactive hyperemia (about 60 mm Hg or lower) led to decreases in the cerebral venous pO_2 below 20 mm Hg There was usually a very good agreement between changes in CBF, as measured with the clearance methods, and changes in the venous oxygen tension.

There were no indications that the different forms of anaesthesia used, or the differences in the arterial CO_2 tensions, had any significant influences on the main conclusion reached on this or in the following paper (Kjallquist, Siesjo and Zwetnow, this volume)

Discussion

The present experiments were devised to study the effects of increased CSF pressure on cerebral venous pO_2, pCO_2 and pH, and on the venous lactate and pyruvate concentrations The experiments confirmed previous findings, that reductions of the cerebral perfusion pressure below about 60 mm Hg were accompanied by a decreased CBF, and as a rule followed by a reactive hyperemia (Noell and Schneider 1948, Haggendal *et al* 1967) Such reductions in the cerebral perfusion pressure usually gave a lowering of cerebral venous pO_2 to values below 20 mm Hg (see also Troupp *et al* 1967), a lowering of pH, and increases in pCO_2, in the lactate and pyruvate concentrations and in the lactate/pyruvate ratios of the venous blood The changes in pO_2 pCO_2 and pH reflect a decreased oxygen availability and a CO_2 accumulation, secondary to a decrease in the CBF The changes in the venous lactate and pyruvate concentrations probably reflect an increased glucolysis in the tissue, while the increased lactate/pyruvate ratios suggest that this glucolysis is anaerobic in nature (*cf* Siesjo, Kjallquist and Zwetnow 1968 Granholm Lukjanova and Siesjo 1968) The changes seen in the venous blood after the intracranial pressure had been released *i e* an increase in the pO_2 and in the pH, and a decrease in the pCO_2 probably only reflect the hyperperfusion of the brain in the period of reactive hyperemia Thus such changes were usually seen only after such pressure periods which did elicit a reactive hyperemia In some experiments however, the venous pO_2 measured after a period of hypertension clearly indicated a hyperemia which was so shortlasting that it was not revealed by the clearance method used It was unfortunately not possible to calculate $CMRO_2$ in the posthypertensive phases due to the non steady state conditions but there were no indications of gross changes in the $CMRO_2$ after the reactive hyperemia had subsided

The chemical changes observed during decreases in the cerebral perfusion pressure within the autoregulation range (see Fig 2) were rather small and were not regularly observed The findings have been stressed however, since analyses on *cisternal cerebrospinal fluid have shown that* a *transient lactacidosis is observed* under identical conditions (Kjallquist Siesjo and Zwetnow 1969) The anaerobic glucolysis which these measurements show may be the results of a very shortlasting decrease in CBF at the beginning of the period of increased intracranial pressure

It has been reported that reductions of the cerebral perfusion pressures to 30—40 mm Hg are unaccompanied by measurable reductions in the cerebral oxygen consumption (Haggendal *et al* 1967) The present results have shown that such reductions of the cerebral perfusion pressure are accompanied by clearcut changes in the cerebral venous pO_2 pCO_2 and pH in the lactate and pyruvate concentrations and in the lactate/pyruvate ratios However once a normal perfusion pressure has been

restored, analyses on cerebral venous blood will not reveal that a period of reduced flow, and possibly of cerebral hypoxia, has passed unless the analyses are carried out in the period of reactive hyperemia Then, a high venous pO_2 may reflect the hyperperfusion

The longlasting hyperemia of brain tissue has been called 'luxury perfusion' by Lassen (1966), who has also discussed its clinical counterparts In the present experiments the hyperemia was of varying but generally shorter, duration than in a previous work (Haggendal *et al* 1967) The difference is probably attributable to several factors such as pressure periods of shorter durations, and a more superficial anaesthesia in the present experiments The present results do not reveal the factors responsible for the hyperemia, but they indicate that it is preceded by a period of critically decreased oxygen availability, increased concentrations of CO_2 and lactic acid and by signs of tissue hypoxia (increased lactate/pyruvate ratio) The possible coupling between these metabolic changes and the subsequent reactive hyperemia will be discussed in a following paper (Kjällquist Siesjo and Zwetnow 1969)

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Studies of Blood-pressure Regulation I. Common-carotid-artery Clamping in Studies of the Carotid-sinus Baroreceptor Control of the Systemic Blood Pressure

By

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Abstract

Öberg, P Å and U Sjostrand *Studies of blood-pressure regulation I Common carotid artery clamping in studies of the carotid-sinus baroreceptor control of the systemic blood pressure* Acta physiol scand 1969 75 276—286

A method has been worked out by which one of the closed control circuits in the blood pressure regulating system in the dog can be reversibly interrupted for varying periods of time Careful studies of the chemistry of the carotid sinus blood using this method as well as of other conditions described in the literature make it probable that the chemoreceptor mechanism in the carotid sinus region and the C N S remain unaffected in this connection The method provides the pre requisite conditions for on line simulation of the regulatory properties of the carotid sinus reflex as regards the baroreceptor function

In connection with investigations of the regulatory properties of the carotid sinus reflex on the blood pressure it became necessary to create a step function in the intrasinusal pressure in order to secure a pure baroreceptor effect in the carotid sinus region. The present article deals with a method by which reproducible step functions in pressure can be obtained for long intervals of time by clamping the common carotid arteries with insignificant reflex chemoreceptor effect on the systemic blood pressure

Other authors who in this connection have related the impulse activity of the sinus nerve with the intrasinusal pressure (Koch 1929 Landgren 1952) have isolated the carotid sinus and its associated vessels from their connections with the rest of the vascular system and produced a step function by a transient change of pressure in the perfusion fluid used (Stegemann *et al* 1966) However with this method there are technical difficulties in securing a viable preparation The method has

therefore often been simplified by producing the change in intrasinusal pressure by bilateral clamping of the common carotid arteries

The disadvantage of the clamping method is that the step function which is obtained by clamping the common carotid arteries does not have a perfect form, particularly during long periods of time The reproducibility is also unsatisfactory, especially as regards the amplitude It has been observed (Schmidt 1932) that, on clamping the common carotid arteries in the dog, the intrasinusal pressure falls initially to about 64 per cent of the normal value but on account of the back flow, especially via the occipital arteries and the internal carotid arteries is built up relatively rapidly (30 sec) to 81 per cent of the normal value Similar investigations have been carried out by Chungcharoen *et al* (1952) and Wang *et al* (1952), with corresponding results, showing that the largest back flow to the sinus region, when the common carotid arteries are clamped, is obtained via the occipital arteries

In all studies of the peripheral blood pressure regulating mechanism it is essential that the circulation in the C N S should be kept intact, so that no central regulatory influence is at work Schmidt (1932) studied the effect on the cerebral blood flow of occluding the carotid arteries and/or the vertebral arteries and found that on clamping the common carotid arteries bilaterally, the cerebral blood flow in the dog was reduced by, on the average, 45 per cent Chungcharoen *et al* (1952) pointed out that the vertebral arteries in the dog are responsible for a substantial part of the normal cerebral circulation. On clamping the common carotid arteries these authors found that there is a considerable reversed flow to the carotid sinus via the internal carotid arteries, primarily on the basis of the satisfactory blood supply via the vertebral arteries but also via the occipital arteries which communicate with the vertebral arteries

The chemoreceptor activity in the receptors which have been demonstrated in the common carotid arteries and their branches—particularly in the carotid body—and also in other parts of the adjacent vessels has been studied by several authors (for review see Torrance 1968) Schmidt (1932) found on dogs that even if the occipital arteries were ligated at a distance of 5—10 mm from the carotid arteries a chemical reflex was still obtained but if the occipital arteries were ligated nearer the carotid region the chemical reflex was entirely absent. The change in the chemoreceptor stimulation on clamping the common carotid arteries has been discussed by Euler and Liljestrand (1936) who pointed out that there are both respiratory and blood pressure effects on the basis of a changed chemoreceptor stimulation Winder (1933) Neil (1951) and Chungcharoen *et al* (1952) discussed the significance of the blood flow through the carotid body in connection with the clamping of the common carotid arteries

Methods

The experiments were carried out on four mongrel dogs of both sexes weighing between 15 and 27 kg (the dogs were between nine months and two years old) After the i v injection of an initial anesthetic (Pentothal sodium® Abbott Laboratories Ltd Queenborough England), 20 mg/kg b w and intubation there followed the intermittent intravenous administration of 0 8 % chloralose (E Merck AG Darmstadt West Germany) at body temperature With

Arrangement for aa car comm clamp ng & sino–ven shunt ng

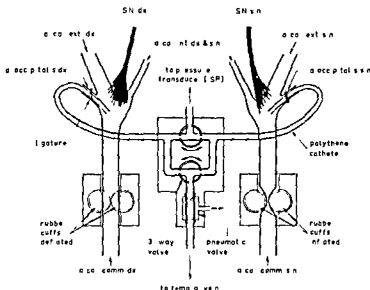


Fig 1 Schematic representation of the arrangement for clamping the common carotid arteries together with sino venous shunting and intrasinusal pressure measurement For further in formation see the text

spontaneous ventilation (room air) it has been shown in separate studies (Danielson *et al* 1969) that with adequate depth (stage III) this form of anesthesia produces a normal acid base status in arterial blood (actual pH 7 38—7 40) and has a minimal effect on the autoregulation of the kidney (Grangsjo 1969) This anesthetic does not alter the response of the vasomotor centre to electrical stimulation of the sinus nerve in the dog (Neil *et al* 1949) The vessels in the two carotid sinus regions were dissected free after which the dogs were heparinized by the intravenous administration of 5 mg/kg body weight of heparin (Vitrum AB Stockholm Sweden)

The common carotid artery was dissected completely free from its connection with the surrounding vessels and nerves for a distance of 10—12 cm below the carotid sinus The superior thyroid artery was ligated and cut off (Green 1967) The external carotid artery was dissected free for 2—3 cm and the internal carotid artery for 1—2 cm above the carotid sinus The anatomical conditions of the baro and chemoreceptors in the dog described by Ask Upmark (1935) and Schmidt (1941) made it possible to carry out the dissection in such a way as to enable stimulation electrodes to be applied to the sinus nerve near its origin in the sinus In this connection it was probable that only the baroreceptor nerves were affected by the
these nerve fibres produced no effect on the respiration
on the other hand there was an effect on the blood
Eyzaguirre and Koyano (1964) stimulated the carotid
chemoreceptor activity was inhibited during the actual stimulation but increased after the stimulation however stimulation of the chemorecepor fibres in the sinus nerve does not produce this type of activity

An atraumatic method of clamping the common carotid artery was used it is shown schematically in Fig 1 The vessels were fixed in a block of perspex in which two rubber cuffs were placed at right angles to the longitudinal direction of the vessel and on both sides
at both common carotid
direct puncture of the
d with a stainless steel
34007 145 AB Stille
Werner Stockholm Sweden) after which the catheter could be fixed by a ligature as near as possible to the point at which the vessel leaves the bifurcation between the external and the

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Unless otherwise stated, the dog breathed air In the studies of the blood chemistry and its relation to carotid clamping carbogens of two types (3 % CO_2+97 % O_2 and 6 5 % CO_2+ 93 5 % O_2) were used during certain periods of the experiments In these cases the endo- ... so as to ensure the
dog had spontaneously
uring period)
e 27, calomel electrode
Copenhagen, Denmark,
inuses (see Fig 1), in

mediately after the sampling By equilibration with 5 % and 12 % CO_2 (+O_2) the P_{CO_2} and the actual bicarbonate could be calculated A separate measurement of the same samples was also made with an oxygen gas electrode (Radiometer type E5046) for the determination of the P_{O_2}

During the experiments the above mentioned parameters were continuously recorded by a UV *recorder* (S E Laboratories UV Recorder S E 2005, S E Laboratories (Engineering) Ltd, London, England) and a data tape recorder (Epsylon Labcorder MR 800A and MR 800A 'Plus Two Unit", Epsylon Industries Ltd, Feltham, England)

Results

The pressure conditions in the right and left carotid sinuses are found to be bilaterally identical

Fig 2 shows characteristic intrasinusal pressures under different conditions in one and the same dog Cases A—D show conditions which are in entire agreement with those previously described in the literature (Chungcharoen *et al* 1952, Wang *et al* 1952), while cases E—H show pressure conditions measured by the shunt arrangement described under "Methods' and illustrated in Fig 1 It is clear from cases A—D that clamping with no shunt produces a greater pressure reduction initially when only the common carotid artery is clamped, both uni- and bilaterally than when both the common carotid artery and the external carotid artery are clamped This fact corroborates the outflow from the carotid sinus region during the clamping of the common carotid artery described by Wang *et al* (1952) In cases A and B, however, the intrasinusal pressure level will gradually increase, owing to the back flow *principally via the internal carotid artery*

In Fig 2 E—H the shunt previously described has been connected By choosing suitable dimensions for the catheters included in the shunt system, an impedance was obtained which made it possible to produce the intrasinusal pressure conditions shown in the figure On connecting the shunt, there is obtained momentarily the desired intrasinusal pressure level, which can be kept constant for a long period of

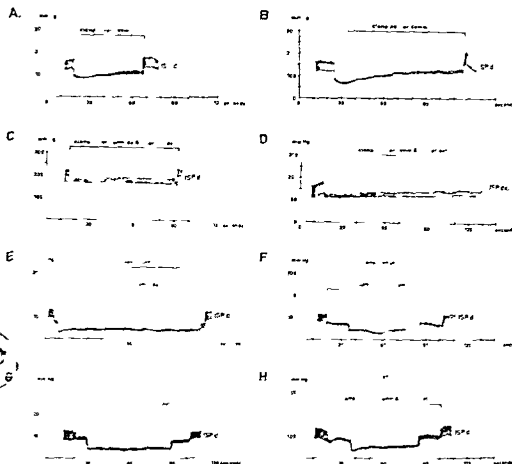


Fig 2 The intrasinusal pressure conditions (mm Hg in the right carotid sinus (ISP_{dx}) under the different normal and clamped and/or shunted conditions described in the text. Abbreviations a.car.comm.dx. = right common carotid artery aa.car.comm. = both common carotid arteries. a.car ext. dx. = right external carotid artery aa.car.ext = both external carotid arteries sino-ven. shunt = sino-venous shunting to femoral vein

time (*a substantial reduction is obtained in the intrasinusal pressure variations synchronous with the pulsations*) As Fig 2 E—H show these conditions apply only during the period for which the shunt is connected

In the experiment illustrated in Fig 3 cases A and B show that this dog had intrasinusal pressure conditions corresponding to those of the dog in the experiment illustrated in Fig 2 Fig 3 C and D show the intrasinusal pressure and the systemic pressure, on the one hand with clamping of the common carotid artery combined with sino-venous shunting and on the other with only clamping of the common carotid artery As is clear on comparing cases C and D the systemic responses are approximately the same in the given intrasinusal pressure functions This is also

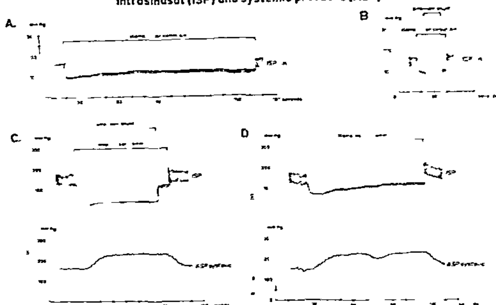


Fig 3 The intrasinusal pressure conditions (mm Hg) in the left carotid sinus (ISP_{left}) under normal and clamped conditions (A) and under normal, clamped and shunted conditions (B) C and D show in the same dog the intrasinusal pressure conditions (mm Hg) in communicating sinuses (ISP) and the systolic systemic blood pressure ($ASP_{systolic}$ mm Hg) under normal clamped and shunted conditions (C) and under normal and clamped conditions (D)

clear merely from Fig 3 C in which the systemic pressure is not changed appreciably when the shunt is cut off while the carotid artery is clamped

In connection with the discontinuance of the clamping of the carotid artery (see for example Fig 4) there appeared practically always a short but clearly recordable bradycardia in the case in which the artery was clamped with no sino-venous shunt (Sigler 1963) This bradycardia was almost never seen when the clamping with the sino-venous shunting was discontinued

Fig 4 shows the respiratory frequency the systolic systemic blood pressure and the P_{O_2} P_{CO_2} actual bicarbonate and pH of the carotid sinus blood under different experimental conditions in one and the same dog (similar results were also obtained for the second dog in which these parameters were measured) Fig 4 A shows the conditions when the common carotid artery was clamped with and without sino-venous shunting (breathing air) Similarly Fig 4 B and C show the conditions in breathing 3 % CO_2 and 6.5 % CO_2 in O_2 respectively The figure only gives the pH measured in the blood which yielded the P_{O_2} and the P_{CO_2} (the continuous pH recording displayed the same level and was not affected by the clamping)

As Fig 4 shows a respiratory stimulation was obtained in breathing carbogen in the form of an increased respiratory frequency compared with breathing air but in no case was any certain change in the respiratory frequency obtained in connection with the clamping As regarded the blood pressure response this

Fig 4 The chemical conditions in the carotid sinus blood the arterial systolic pressure and the respiratory frequency in one and the same dog

A Spontaneous breathing of air

B Spontaneous breathing of 3 % CO_2 in 97 % O_2

C Spontaneous breathing of 6 5 % CO_2 in 93 5 % O_2

was in principle in agreement with the conditions previously described (Fig 3) The recordings of the P_{O_2}, P_{CO_2}, actual bicarbonate and pH show that there are no systematic differences between the non-shunt and the sino-venous shunt cases and that, during the two minutes for which the common carotid artery is clamped, there are no systematic changes in the blood chemistry It should also be pointed out that

unclamping of the common carotid arteries and removal of the sino-venous shunt rapidly produce a blood pressure of the same magnitude as before this procedure This makes it probable that during the clamping shunting procedure the cerebral circulation is sufficient to avoid changes in the blood chemistry of the C N S (as no central regulatory interference can be demonstrated when the supply of afferent information from the biological baroreceptors is restored), i e no C N S medullary ischemic reflex (Guyton and Harris 1951 Guyton and Satterfield 1952) can be demonstrated

Discussion

The arrangement shown in Fig 1 avoided a traumatic effect on the vascular wall which easily produces variations in the systemic blood pressure via the mechanical receptor effect In order to avoid the influence of the baro- and chemoreceptors in the upper part of the common carotid artery (*cf* Boss and Green 1956 Green 1967) the vessels were dissected free as indicated under Methods The shunt was connected to the sinus region by cannulating the occipital artery (Fig 1) for two reasons In the dog the largest back flow of arterial blood with carotid clamping comes through the occipital artery (Wang *et al* 1952) which would affect both the static and the dynamic intrasinusal pressures (the above procedure reverses the flow in the external carotid artery which supplies the sinus region with oxygenated blood by anastomosis with the vertebral artery)

It is known from the literature (Landgren 1952) that with low intrasinusal pressure the vascular wall collapses resulting in baroreceptor activity and that the baroreceptors in the cat have a stimulus threshold for a step function in intrasinusal pressure of about 30—40 mm Hg (Landgren 1952 Ead *et al* 1952) or statically about 60 mm Hg (Koch 1929) In order to obtain intrasinusal pressure conditions with little baroreceptor activity the pressure must be kept within these given limits which could be brought about by adjusting the flow impedance of the sino-venous shunt system in each individual case

A comparison between Fig 3 C and D shows that clamping with and without a sino-venous shunt produces an adjustment to a new systemic pressure level which is equal in both cases As was pointed out above in Fig 3 the circulatory conditions (clamping of the common carotid artery in combination with sino-venous shunting) probably resemble the normal situation more than is the case with only carotid clamping (Fig 3 D) It has already been pointed out that the new systemic pressure level after clamping in the case shown in Fig 3 C—D will be practically the same in spite of the fact that in Fig 3 C the intrasinusal pressure has become considerably lower (below the threshold level) This fact indicates that the pulsation in the intrasinusal pressure have a considerably greater effect on the systemic pressure level than the absolute static pressure a circumstance which has previously been pointed out in the literature (Bronk and Stella 1932 and several others) A check on this is also given in Fig 3 C in which the systemic pressure is retained at unchanged level in

spite of the cessation of the i v shunting but with continued clamping of the common carotid artery *In both Fig 3 C and D there is, on the other hand, a change in the* systemic pressure immediately after the reestablishment of the static and dynamic intrasinusal pressure conditions Thus, Fig 3 C shows the change of systemic pressure on a step function in intrasinusal pressure and thereby constitutes a step-function test of the regulatory system (Öberg and Sjostrand 1969)

As far as the cerebral and medullary circulation is concerned, it can probably be regarded as adequate during the clamping shunting procedure (the pressure conditions produced by Sagawa *et al* (1961) in obtaining cerebral-medullary ischaemia were never present in the experiments described here)

The slightly increased reflexogenic respiratory activity which was demonstrated by Siciliano (1900) and later authors and which is obtained by clamping the common carotid artery (for a survey see Euler and Liljestrand 1936, Heymans and Neil 1958) has been regarded as being caused by a changed chemoreceptor excitation on account of changed conditions in the blood chemistry (Euler and Liljestrand 1943) This *change in the blood chemistry is also supposed to affect the systemic blood pressure* (Euler and Liljestrand 1943, Landgren and Neil 1951) in the cat, for example Chungcharoen *et al* (1952) point out, however, that there is a considerable flow of blood via back flow from the circle of Willis in connection with the clamping of the common carotid artery in the dog as distinct from the cat (Euler and Liljestrand 1943), and therefore the chemoreceptor activity in the dog should be less affected during clamping

The changes in the chemistry of the carotid sinus blood of the cat during clamping of the common carotid artery which were supposed by Euler and Liljestrand (1943) and Landgren and Neil (1951) seem to be irrelevant in connection with the methods used in the present work (*cf* Fig 4 and above) Biscoe and Sampson (1967) point out not only the arterial P_O , the P_{CO_2} and the pH form the basis of the receptor response but also that the receptor response is particularly complex and is dependent on several other factors If the correlations measured by Biscoe and Sampson between the *impulse frequency* and the P_{O_2} and P_{CO}, *respectively* can also be considered to apply in principle to the dog the changes which are seen in the P_{O_2} and P_{CO_2} in connection with clamping in Fig 4 should not cause any obvious change in the pulse frequency in the chemoreceptor fibres which are still intact with the preparation technique described here (*cf* also Eyzaguirre and Lewin 1961)

Euler and Liljestrand (1943) found a systemic-pressure response on clamping the common carotid artery which was greater with a low P_{O_2} value which was later shown by Landgren and Neil (1951) to have been caused by a changed chemoreceptor function The latter authors concluded that on the other hand a central regulatory effect could be precluded Similar changes in the magnitude of the systemic response may perhaps be seen in Fig 4 (5—10 mm Hg greater pressure response in Fig 4 A, compared with Fig 4 B and C) which may have been due to the remaining chemoreceptor function

It should be pointed out however that during the clamping shunting procedure

used in these experiments the cerebral circulation was satisfactory Regardless of the length of the clamping shunting period the mean blood pressure level was the same before and after this procedure The postclamping-shunting pressure level was obtained in a typical and damped oscillatory fashion The relative insensitivity of the baroreceptors to the conditions in the blood chemistry has moreover been demonstrated by Schmidt (1941), amongst others

By way of summary, the method described here—the combination of clamping the common carotid artery and sino-venous shunting—would seem to be an adequate method of opening the closed regulatory loop This method creates opportunities of studying the regulatory properties of the cartid sinus reflex by analog on line computation and of investigating the dynamic properties of the regulatory system from the systemic response (Öberg and Sjostrand 1969)

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Studies of Blood-pressure Regulation. II. On-line Simulation as a Method of Studying the Regulatory Properties of the Carotid-sinus Reflex

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Abstract

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A method has been worked out by which one of the closed control circuits in the blood pressure-regulating system of the dog can be reversibly opened and closed again with the aid of an on line analog computer. This makes it possible to study the regulatory properties of the carotid sinus reflex, as regards the baroreceptor function, in the regulation of the systemic blood pressure. Proportional characteristics in the analog produce marked instability in the blood pressure regulation, while with increasing proportional plus-derivative characteristics the stability is increased. The electronic analog can be given characteristics that produce the same step-function response in the systemic pressure as the biological reflex.

In the mammalian cardiovascular control system the carotid-sinus reflex constitutes one of the most important feedback paths for arterial pressure control. Much attention has therefore been paid to this reflex, ever since it was discovered at the end of the 19th century (for reviews, see Heymans and Neil 1958, Kezdi 1967). The baroreceptor function of the carotid bifurcation and the afferent nervous activity in the sinus nerve under different intrasinusal-pressure conditions have been especially examined (Bronk and Stella 1932, Ead *et al.* 1952, Landgren 1952, Spickler and Kezdi 1967).

In recent years some authors have analysed the blood-pressure regulating properties of the carotid sinus reflex, using methods according to control theory (Guyton *et al.* 1951, Warner 1958, Clynes 1962, Scher and Young 1963, Levison *et al.* 1966, Milhorn 1966, Milsum 1966, Stegemann *et al.* 1966, Spickler and Kezdi 1967).

It has been shown that the baroreceptors in the carotid-sinus region are sensitive not only to the mean intrasinusal pressure but also to the rate of change in this

pressure that is the afferent sinus nerve activity is proportional to both the mean pressure and to the derivative of the pressure (Bronk and Stella 1932) It has also been shown that pulsatile intrasinusal pressure is more effective than non pulsatile with the same mean pressure in the reflex inhibition of the vasomotor centre (Ead *et al* 1952, Gero and Gerova 1962) In the normal regulatory system the frequency of the afferent nervous activity in the sinus nerves is at its maximum during systole and the beginning of diastole (Bronk and Stella 1932 Landgren 1952)

Control systems of this type are called proportional plus derivative (P+D) systems This type of control is often chosen in technical systems to prevent instability following transient disturbances in the system (Milhorn 1966, Milsum 1966)

In order to study the proportional puls derivative action of the carotid sinus reflex and its dependence on the mean pressure and the derivative of pressure in the regulatory system an electronic analog of the reflex was built and used in on line operation i e constituting a part of the biological regulatory loop in dog experiments

The fundamental principles of these experimental studies were as follows

1 The studies were carried out on the dog because its anatomy is advantageous in this respect (Öberg and Sjostrand 1969) and the functioning of its vasomotor centre is relatively little affected under chloralose anesthesia (Neil *et al* 1949) in comparison with sodium pentobarbital (Guyton and Harris 1951)

2 The cardiovascular conditions in the experimental animal under chloralose anesthesia (the anesthetic generally used in circulation studies) must be regarded as typical of the cardiovascular function

3 A simplified electronic analog of the baroreceptor function in comparison with the intact function in the carotid sinus region of the experimental animal can yield information about the functional importance of the baroreceptor reflex particularly as regards the dynamics of the blood pressure regulation

4 In order to study the stability of the blood pressure regulating system with a changed baroreceptor function it is necessary that the analysis should be performed by a closed loop procedure i e in the way described in paragraph 5 below

5 The closed biological regulatory loop can be broken by a special procedure (Öberg and Sjostrand 1969) and the electronic analog can be connected to the afferent nerve paths of the biological control loop which means that the loop is re established This procedure gives great freedom to modify the pulse position modulated (ppm $\approx$ frequency modulated) afferent information by varying the different static and dynamic parameters of the analog

6 In this connection the carotid sinus reflex may be regarded as a two port network (black box) whose input signal (the intrasinusal pressure) produces a pulse position modulated signal and whose output signal is the systemic pressure This means that all other receptors feedback chains and effector organs together with the experimental procedures are regarded as belonging to the two-port network By this approach using a reduced system (lumped parameters) the system can be given a simple mathematical form

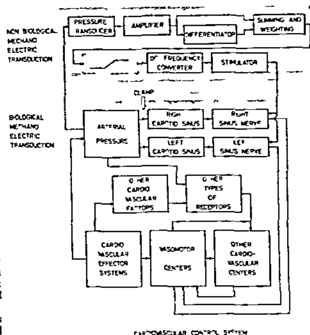


Fig 1 Block diagram of the cardiovascular control system with the normal carotid sinus-reflex arcs indicated as Biological mechano-electric transduction The electronic analog system is indicated as Non biological mechano-electric transduction

The cardiovascular system can be schematically represented in many different ways depending of which part is in the focus of interest The system outlined in Fig 1 can be regarded as being composed of two functionally separable blocks (1) the biological mechano-electric transduction part, which can be replaced intermittently by an electronic (non biological) analog and (2) the rest of the complete cardiovascular system that is structurally and functionally intact This paper presents the principles and experimental methods for qualitative studies of the proportional plus-derivative action of the carotid sinus reflex and its role in the baroreceptor control of the systemic blood pressure

Methods

The experiments were performed on 12 mongrel dogs of both sexes weighing between 15 and 27 kg (their ages never exceeded 2 years) They were anesthetized in the way described in a previous article (Öberg and Sjostrand 1969) In this connection chloralose was used—an anesthetic which is commonly used in studies of the central and peripheral circulation (see for example Neil *et al* 1949 Scher and Young 1963 Kezdi 1967) and which does not produce any appreciable disturbances in the acid base balance (Danielson *et al* 1969) This form of anesthesia also has minimal respiratory effects and the endotracheally intubated dogs spontaneously breathed room air during the experiments The vessels in the two carotid sinus regions were dissected free in the manner previously described (Öberg and Sjostrand 1969) When the preparation was complete the dog was heparinized (Öberg and Sjostrand 1969)

A previously described method was used in order to momentarily exclude the biological baroreceptor function in the carotid-sinus region and to retain this breaking of the control loop for several minutes without affecting the rest of the system (Öberg and Sjostrand 1969) The electronic analog (see below) generated the afferent information by electrical stimulation of the exposed nerve fibres from the carotid sinus receptors as already described (Öberg and Sjostrand 1969)

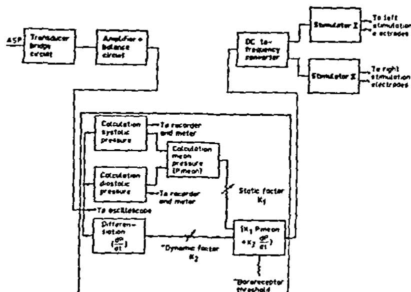


Fig 2 Block diagram of the electronic analog system.

During the experiments the respiratory frequency and the relative depth of the respiration were recorded by a thermistor connected to the endotracheal tube The systemic blood pressure was measured by a Statham P23AC transducer (Statham Hato Rey Puerto Rico) via a thick and rigid catheter inserted in the femoral artery with its orifice on a level with the middle of the iliac artery This transducer was connected to an amplifier system and produced recordings of the systolic and diastolic systemic blood pressure and the signal was also conditioned by the electronic analog In all figures in which the systemic blood pressure is shown the systolic pressure has been chosen as being representative (see footnote in the section on "Experimental Procedure p 6 The intrasinusal pressure was measured by a separate P23AC pressure transducer (Öberg and Sjostrand 1969)

During the experiments the above parameters were continuously recorded by a UV recorder (S E Laboratories UV Recorder S E 2005 S E Laboratories (Engineering) Ltd London England) and a data tape recorder Epsylon Labcorder MR800A and MR800A "Plus Two Unit Epsylon Industries Ltd Feltham England

Apparatus

The block diagram of the carotid-sinus simulator is shown in Fig 2 The systemic blood pressure is measured with the aid of an arterial pressure transducer (Statham P23AC) The unbalance voltage which arises in the transducer bridge is amplified by two operational amplifiers of the Nexus SA 3a type Nexus Research Lab Inc Canton U S A the first of which is differentially coupled. The amplifier chain has a linearity of 0 5 % and very good long term stability

After amplification the signal is fed partly to an oscilloscope for control purposes and partly to 3 computing circuits The systolic and diastolic pressures are calculated in a similar way by conventional circuits of the peak value-sensing type The operational amplifier Nexus SQ 10a is used throughout these circuits The time constants of these circuits were adapted to the dynamic states which occurred in the experiments described here The calculated systolic and diastolic pressures were displayed on indicator instruments graduated in mm Hg

Then the derivative of the systemic blood pressure was calculated by mechanizing the transfer function

$$H = \frac{k \cdot s}{1 - \tau \cdot s}$$

where $\tau = 0.007$ sec s = the Laplace operator and k = a constant This method of derivation yields good stability and satisfactory accuracy within 1 % with the frequency spectrum contained in the arterial pulse wave (Ruch and Patton 1965

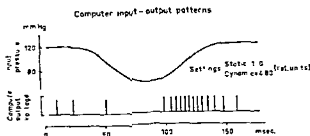


Fig 3 The response of the electronic analog to a test signal

The mean systemic pressure is obtained from the calculated values of systolic and diastolic pressure The definition of mean systemic pressure (see *e g* Guyton 1966) has been given different mathematical interpretations in the literature (see, for example, Wiggers 1944, Berne and Levy 1967) and may be formally expressed as follows

$$P_{mean} = P_{diastolic} + \frac{1}{T}\int_0^T P(t)\,dt,$$

where T = the duration of the cardiac cycle and P(t) = the time lapse of the arterial pulse curve The integral part of this expression can be approximated (triangle approximation) to

$(P_{systolic} - P_{diastolic}) / \sqrt{2}$, i e

$P_{mean} \approx P_{diastolic} + (P_{systolic} - P_{diastolic}) / \sqrt{2}$

This mean systemic pressure is very suitable for calculation by simple analog circuits

The characteristics of the systemic blood pressure, i e the mean pressure and the derivate of the pressure, obtained in this way are then summed in a summation circuit, in which the magnitudes of these two variables can be varied independently of each other The threshold value characteristic of the baroreceptors is also simulated in this circuit It was chosen in accordance with the unambiguous data found in the literature (for a survey, see Öberg and Sjostrand 1969)

Thus in the output of the above summation circuit there is the sum of the weighted parts of the mean blood pressure and the [illegible] is applied to a linear DC-frequenc[illegible] and Sjostrand 1968) This value [illegible] values measured in a single fibre [illegible] output signal of the DC-frequency converter controls 2 stimulators one for each sinus nerve The signals from the stimulator outputs which have low impedances and are insulated from ground had durations of 0 1 msec, and amplitudes of 2 V in the experiments—values which were found to be suitable in the checking experiments carried out beforehand A delay circuit is used so as to displace the phases of the stimulator output signals by 0 15 msec in relation to each other This method of stimulation was found to be necessary in order to prevent the cross-stimulation between the right and the left electrode which otherwise arises (Warner 1958 and which produces undesirable effects, such as contractions of the laryngeal musculature In the circuit used here this is prevented by the fact that the stimulation pulses on the two sides are never applied simultaneously

An example of the relationship between the input signal and the corresponding output pulse train is shown in Fig 3 (the "static" setting = 1 1 and the "dynamic = 4 8)

The relationship between the mean blood pressure and the mean pulse frequency of the system described in Fig 2 is shown in the left-hand part of Fig 4 for some selected settings The [illegible] five [illegible] var [illegible]

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circled the sinus nerve like a snare The bands which were covered with plastic insulation on their outer sides could be thightened and locked by a special screw device in such a way that the nerve and the silver band were in good atraumatic contact with each other

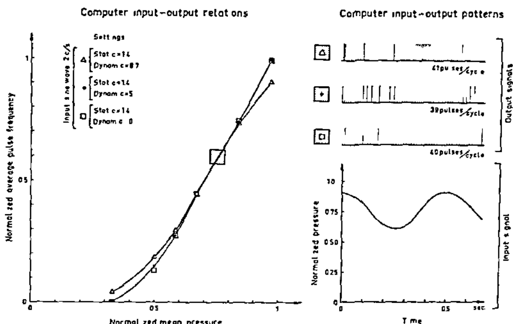


Fig 4 The relation between the mean blood pressure and the mean pulse frequency (sinusoidal input signal) for the electronic analog at three different parameter settings is shown in the left hand part of the figure, while the right hand part shows the impulse pattern obtained with the corresponding parameter settings from a given sinusoidal input signal with the mean pressure = 0 75 normalized units

Experimental procedure

The normal dynamic response measured as the systemic arterial blood pressure, of the intact ... n carotid system ... (including also ... blood pressure ... After 1—2 min ... vious level with a slight initial oscillation This is regarded as the normal regulatory response Again the common carotid arteries are clamped and as soon as the rate of arterial pressure elevation is almost zero the electronic analog is connected on line The regulatory response in the systemic arterial pressure is studied for different parameter settings When the clamps are removed the arterial blood pressure level should now go back to the level existing before clamping if the cardiovascular regulatory system is unchanged (no functional disturbances of vasomotor centres etc, *cf* Öberg and Sjostrand 1959) The normal regulatory response was intermittently checked during the experiments but was usually unaltered for several hours The main disturbances in the normal regulatory response were usually due to general anesthesia and appeared shortly after the injection of chloralose (probably depending, among other things on the direct local effect on the baroreceptors (Neil *et al* 1949 Biscoe and Millar 1964)) For this reason regulatory studies were never made within a 5 min period after iv chloralose injections

[1] Recordings of both systolic and diastolic pressures showed that, in these experiments it was sufficient to record one of them as they both contained the same information about the principle regulatory properties of the blood pressure regulatory system

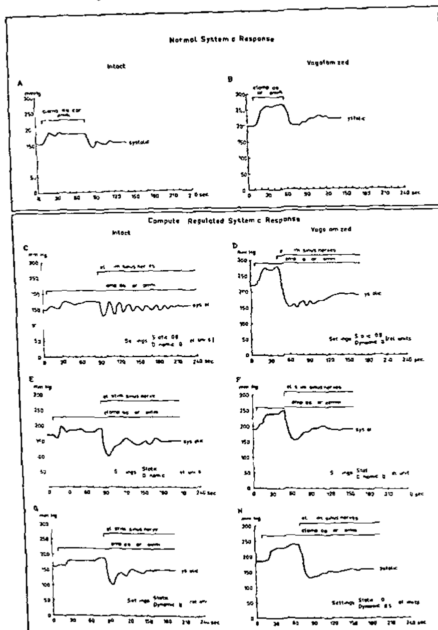


Fig 5 Systemic responses measured as systemic systolic blood pressure under different conditions Graphs A C E and G show the results in the intact dog while graphs B D F and H show the corresponding results in the vagotomized case A The normal systemic response of the systolic blood pressure) to clamping of both carotid arteries combined with sinovenous *shunting* (indicated only as "clamp aa car comm) Note that stable levels are reached only after a slight *oscillation* *C E* and *G* The computer regulated response under different computational conditions with clamping and shunting as in A These graphs show that with an increasing dynamic term ("dynamic) the stability of the systemic response is increased B The normal systemic response as in A but in the vagotomized case (same dog) *D F and H* As in *C E* and G but in the vagotomized case showing essentially the same response as in the intact dog

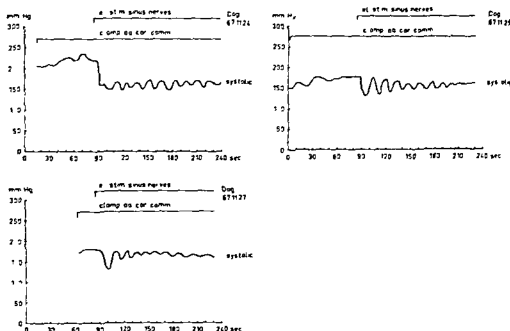


Fig 6 The computer-controlled systemic blood pressure responses in three different dogs in the series with settings for "static" of 0 8 and for "dynamic" of 0 See the text for further details

Results

Studies of blood pressure regulation were carried out on 12 dogs and some results have been selected as being typical and thereby representative of the whole material

Fig 5 A shows the normal response to common-carotid-artery clamping and sinovenous shunting (note that the time scale in Fig 5 is different from that of Fig 4) The systemic response to a positive intrasinusal pressure step function will be regarded as the normal regulatory response of the intact dog

After clamping and shunting and the rise of the systemic pressure the analog is connected on line (the systemic response demonstrated in Figs 5 C E and G Fig 5 C shows what happens if no pressure derivative is introduced into the artificial system. Hunting occurs with a period time of about 20 sec (in this experiment the settings are mean pressure ("static") 0 8 and derivative ("dynamic") 0 measured as relative units) This case corresponds to a purely proportional control system and instability appears when the dynamic term is set at zero

In Fig 5 E a pressure derivative factor of two relative units is introduced and thereby the analog of the carotid-sinus reflex has proportional-plus-derivative characteristics The result of adding this dynamic term is a more damped response If this term is increased still more (as in Fig 5 G) the systemic pressure response is

rather damped (almost similar to the natural one (Fig 5 A)) As a summary account of all these figures together it can be stated that with an increased dynamic term the stability of the regulatory system is increased although the number of stimulation pulses per cardiac cycle is approximately the same at all the different settings (*cf* Fig 4) What differs is the pattern of the pulses *i e* the pulse distribution with time

In Fig 5 B D F and H recordings of the systemic blood pressure are shown after both the vagi have been cut (vagotomy 2—3 cm below the carotid bifurcation) The response to the positive step function in intrasinusal pressure (Fig 5 B) is very damped and is regarded as the normal regulatory response in the vagotomized dog

The systemic responses in the computer regulated cases (Fig 5 D F and H) have essentially the same tendency as in the intact case It should be pointed out that in Fig 5 D oscillations occur like the ones in Fig 5 C at roughly the same mean systemic pressure level The computer regulated reference level (Fig 5 D F H) is essentially the same as in the previous experiments in this figure (Fig 5 C E G) Compare also the results obtained in hypertensive dogs with sinus nerve stimulation by Griffith and Schwartz (1964) The responses in general in three hypertensive dogs of the experimental series were markedly damped

Fig 6 shows the computer regulated systemic responses in three dogs in the experimental series with parameter settings of 0 8 (static) and 0 (dynamic) relative units The recordings show clearly the undamped character of the purely proportional control system It should be pointed out that the reference level after the connection of the computer on line in all three cases is approximately the same in spite of the different systemic pressure levels when the computer was connected In its turn this can be interpreted as meaning that the information transmitted from the computer to the vasomotor centre in these three experimental animals was relatively homogeneous (provided that the black box parts of these animals can be regarded as being similar) This accordingly indicates that the electronic analog is an effective part of the closed control circuit in on line simulation (see also under Discussion) Another noteworthy circumstance is the obvious equality of period time in the oscillations with this parameter setting with $T \approx 20$ sec—a value which is in good agreement with that given by Guyton and Harris (1951)

Discussion

General methodological problems

For the reasons given above chloralose was regarded as the anesthetic which would probably have the least effect on the systemic blood pressure This view was also supported by a statement by Neil Redwood and Schweitzer (1949) No evidence has been found in these animals (rabbits and dogs) that chloralose causes any modification of blood pressure response to carotid sinus nerve stimulation In all circumstances a depressor response to electrical stimulation of the sinus nerve was obtained indicating that the effects of baroreceptor fibre st mulation are predominant over those of chemoreceptor fibre stimulation

The conditions that prevail during clamping of both common carotid arteries combined with sino-venous shunting have been thoroughly discussed in a previous article (Öberg and Sjostrand 1969) In this connection the intrasinusal step function in pressure has been discussed in relation to the carotid-sinus baroreceptors i e to what extent only the baroreceptor chain in the carotid-sinus reflex is disconnected during the clamping and shunting period. The clamping and shunting procedure can probably be regarded as producing a selective and complete baroreceptor disconnection

In the previous article just mentioned a method of stimulating electrically the afferent nerve paths of the carotid sinus baroreceptors has been described and discussed Studies of the systemic response (measured by the systemic blood pressure level) showed that the optimal effect of the electrical stimulation of the afferent baroreceptor fibres was obtained with an amplitude of 2 V and a duration of 0 1 msec in the stimulation pulses As regards order of magnitude this agrees with the statements of Bilgutay *et al* (1964) for example As regards the duration of the stimulation pulses somewhat different data have been given by Neil *et al* (1949) However it may be reckoned that absolute values are difficult to give as the volume conductivity and the cable properties greatly influence the individual case (*cf* also Griffith and Schwartz (1964))

The possibilities of selectively disconnecting the baroreceptors in the carotid sinus region and of selectively stimulating the afferent nerve fibres of these baroreceptors electrically create the pre requisite conditions for connecting an electronic analog which can then take over the function of transferring information between the pressure and the afferent nerve paths from the baroreceptors In this connection the analog can be given definite characteristics such as a fixed threshold value as well as clearly defined static and dynamic characteristics These characteristics will accordingly represent the complex biological system.

Owing to the design of the experimental method the intrasinusal pressure cannot be used as a control signal for the electronic analog (Öberg and Sjostrand 1969) The systemic pressure is therefore measured by a catheter with its orifice in the middle of the iliac artery in which the arterial pulse wave does not have exactly the same appearance as in the carotid sinus (Hamilton and Dow 1939)

The electronic analog

In developing the electronic analog used in this work, the aim was to design it so as to simulate in essential points the basic properties of the baroreceptors known from biological measurements By this means the potentialities of the method used could be evaluated from experimental experience The characteristics of the analog used have been given above in the section on Apparatus Characteristics which this analog lacks are *e g* adaptation properties (*cf* Bronk and Stella 1934 Barany 1943 Landgren 1952 Biscoe and Millar 1964 and different degrees of sensitivity to positive and negative pressure derivatives *cf* Landgren 1952 Clynes 1962 Christensen *et al* 1967)

The previous described definition of mean blood pressure was constructed on the basis of a triangular approximation of the arterial pulse wave It agrees well with other definitions of mean blood pressure described in the literature

Derivation of analog signals is a complicated electronic problem From the many electronic derivation principles described in the literature, a type was selected, on the basis of direct measurements in the circuits concerned in which the above-mentioned transfer function is mechanized By choosing a suitable value of *t* for the actual signal, very satisfactory accuracy can be obtained

The experimental procedure

Owing to the design of the experimental procedure, it is easy to check intermittently during the course of the experiment the step-function response of the biological control system. This is a valuable feature to facilitate checking on any changed experimental conditions (effects of anesthetic or acid base balance etc) This check is not possible in experimental procedures which include the isolation of the carotid bifurcation and the associated vessels On the other hand, the amplitude of the step function in intrasinusal pressure obtained by the method applied here is dependent on the systemic blood pressure

In the intact experimental animals the afferent paths from the aortic-arch receptors are intact and their function is considered to belong to the 'black box" part of the blood pressure regulating system Their function in the black box part probably has a damping effect on transient states in the controlled variable (systemic pressure) In the vagotomized experimental animals the afferent paths of the aorta arch and pulmonary vessel receptors (Bianconi and Green 1959) are broken and the experimental animal s systemic blood pressure level is, as a rule, considerably raised In this case the damping can probably be explained by the high systemic pressure level (see the section on Results as regards the hypertensive experimental animals)

Control theory aspects

Many authors have previously investigated the effect of the intrasinusal pressure on the afferent impulses in the sinus nerve from the carotid sinus baroreceptors (Bronk and Stella 1932 Schmidt 1933, Landgren 1952 Gero and Gerova 1962 Spickler and Kezdi 1967 and several others) Other authors have studied the effect of the intrasinusal pressure on the systemic blood pressure (Levison *et al* 1966 Stegemann *et al* 1966) In this connection it has been found that a dynamic intrasinusal pressure is more effective than a static intrasinusal pressure in the reflex control of the systemic blood pressure

One author (Warner 1958) has previously used an electronic analog in studies of the carotid sinus reflex However his method did not involve the opening of the closed control loop The afferent information supplied via the sinus nerves consisted of both signals of biological origin (the baroreceptors) and signals which had been superimposed by electrical stimulation controlled by the electronic analog

Warner s results are difficult to interpret as the analog did not replace a part of the natural reflex path which reduces the possibilities of studying the significance of the pressure derivative, for example

In order to investigate experimentally the stability of the systemic blood pressure and its dependence on static and dynamic intrasinusal pressures it is necessary to study the pulse position modulated afferent information supplied to the vasomotor centre, which can be done advantageously with an electronic analog By this on line technique it may also be possible to simulate conditions in the control loop which are not to be regarded as normal physiological conditions but which yield increased information about the general properties of the control system

In order to study the static and dynamic properties of the control loop simply the electronic analog was designed in such a way that a certain number of stimulation pulses per unit of time always corresponded to given mean blood pressures independently of the dynamic parameter setting as is shown in Fig 4 This means that the number of output pulses per cardiac cycle was always the same for a mean pressure setting but the pulse distribution with time varied with the dynamic term of the analog (this is in complete accordance with experimental findings for the carotid sinus baroreceptors by Ead *et al* (1952) and for the aortic arch baroreceptors by James (1968) The parameter setting of the analog influences only the derivative of the frequency *i e* the frequency modulation of the output signal The relationship between the static and dynamic parameters is of importance for the stability and their sum is of importance for the regulated level of the systemic blood pressure i e on line simulation as pointed out in the section on Results

The conclusion which could be drawn with the guidance of the results in Fig 6 (see above) was that the electronic analog was an effective part of the closed control loop In comparing the intact and vagotomized cases in Fig 5 it may be observed that the percentage reduction in systemic blood pressure in the computer regulated case is greater in the vagotomized dogs than in the intact dogs and this fact is accentuated by increasing dynamic characteristics in the electronic analog —A greater percentage reduction in blood pressure with electrical stimulation of the sinus nerve in dogs which had been made hypertensive by the Goldblatt technique in comparison with normotensive dogs has been reported by Griffith and Schwartz (1964)

From the results of the experimental series it is clear that the blood pressure regulating system is of the second order a fact which has previously been discussed in the literature by making linear approximations (Warner 1958 Scher and Young 1963 Levison *et al* 1966) The coefficients of the system may quite generally be said to be non linearly pressure and/or time dependent and dependent on the afferent impulse coding An increased degree of modulation produces increased stability in the system The period time of the oscillations varies in this connection (which also indicates the non linearity (and/or time dependence) of the system) and had a T≈20 sec, which agrees well with the value for which the blood pressure regulating system of the dog has maximal amplification according to Levison *et al* (1966)

As a substitute for or a complement to conventional pharmacological methods of lowering the blood pressure of patients with hypertension, electronic 'baropacers' (Griffith and Schwartz 1964, Bilgutay 1964) have recently been introduced for experimental use in patients (or experimental animals, Myers *et al* 1968), i e implantable stimulators, with the stimulation electrodes placed above the carotid sinus or sinus nerve on one or both sides These 'baropacers" work more or less on the open loop principle, which may, however, be supposed to be unsatisfactory from the point of view of control theory Bearing in mind the practical difficulties associated with baropacer therapy, the results presented in this and subsequent more quantitative articles can probably be used as direct guidance in both the technical and the practical development of 'baropacer technique

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The Action of Tetrodotoxin on the Frog's Isolated Muscle Spindle

By

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Abstract

E. X. Albuquerque, S H Chung and D Ottoson *The action of tetrodotoxin on the frog's isolated muscle spindle* Acta physiol scand 1969 *75* 301—312

Tetrodo[illegible] of the [illegible] Develc[illegible] delay [illegible] action [illegible] synchronization of the conducted activity in the branched afferent terminals After block by tetrodotoxin for up to 20 min the normal response could be restored by washing the preparation with Ringer's solution The blocking action of tetrodotoxin was delayed and reduced by increasing calcium, removal of calcium had the opposite effect. Tetrodotoxin ($\leq$1000 ng/ml) had no significant effect on the receptor potential With low sodium plus tetrodotoxin the receptor potential was reduced by about 50 % this reduction was reversed by addition of normal sodium It is suggested that the sodium carrying system of the generator potential differs from that underlying the regenerative activity

Tetrodotoxin has been found to provide a new and interesting tool for studies of the ionic mechanisms underlying the excitatory processes in nerve and muscle A specific action of the toxin is to block the passive sodium transport (Narahashi *et al* 1960 Nakamura, Nakajima and Grundfest 1965, Moore and Narahashi 1967) There is however, conflicting evidence with respect to the action of the toxin on the receptor potential It has been reported that the toxin blocks impulse activity without affecting the receptor potential in the slowly adapting stretch receptor of crayfish (Loewenstein Terzuolo and Washizu 1963) and of lobster (Albuquerque and Grampp 1968) Loewenstein *et al* (1963) also reported that the receptor potential of the Pacinian corpuscle was not appreciably affected by the toxin In two subsequent studies it has been found that the toxin caused up to 40 % reduction of the generator potential of the Pacinian corpuscle (Ozeki and Sato 1965, Nishi and Sato 1966) A reduction

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of the receptor potential of the Pacinian corpuscle by tetrodotoxin may be interpreted as a consequence of the blocking action of the sodium carrier mechanism involved in the generation of the receptor current (*cf* Diamond, Gray and Inman 1958) On the other hand, experiments in which tetrodotoxin failed to affect the receptor potential (*cf* Loewenstein *et al* 1963) could be explained by assuming that in these systems a separate sodium carrying system exists for the generator current

The present investigation was undertaken to examine the interaction of tetrodotoxin with the sodium carrying system of the generator potential of the muscle spindle of the frog It has been shown that sodium ions are responsible for approximately 60 % of the transport of charge across the receptor membrane of the spindle during the response to stretch (Ottoson 1964) If tetrodotoxin blocks sodium transport of the generator current of the muscle spindle, this portion of the response should be eliminated and a subsequent removal of sodium from the external solution would not cause any further reduction In such an event, the toxin would also affect the impulse generation mechanism of the muscle spindle afferent Since calcium ions are involved in the processes underlying membrane excitability (Frankenhaeuser 1957, Frankenhaeuser and Hodgkin 1957) and also play an important role in the ionic processes connected with transducer actions of the sensory membrane of muscle spindle (Ottoson 1965) it was considered worth while to examine the possible interaction of this ion with the action of tetrodotoxin

Methods

Experiments have been performed on single muscle spindles from the *m ext dig long IV* of the frog (*Rana temporaria*) Isolation and mounting of the preparation were carried out as described in earlier papers (Ottoson 1961 1965) The spindle was fastened between two fine nylon rods in a small chamber The points of attachment were near the polar ends of the spindle The length of the spindle was adjusted by taking up slack until any further increase in length would cause an increase in the spontaneous firing rate recorded from the nerve Techniques of delivering stretches of varying amplitudes and velocities have been discussed in detail elsewhere (Ottoson 1965 Shepherd and Ottoson 1965) The length of the mounted [illegible]

The Ringer's solution used in most experiments had the same composition as that used by Adrian (1956) Calcium free solutions were prepared with EDTA (5 mM) Solutions with [illegible] Ringer's solution of the following millimolar [illegible] um free solutions were obtained by re[illegible] asis [illegible] obtained from Sankyo Company Ltd Tokyo Japan For each experiment 1 mg of the drug was dissolved in 10 ml of deionized distilled water Further dilutions were made in the physiological solution Concentration of the toxin is expressed in g/ml

Results

A Effect of tetrodotoxin on the impulse response to stretch

When the spindle was exposed to tetrodotoxin at concentrations of $0.8-1 \times 10^{-7}$ g/ml the action potential in response to stretch was usually eliminated within 2–3

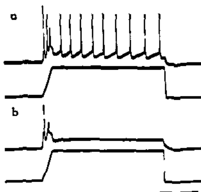


Fig 1 Blocking of static discharge with tetrodotoxin Records of response to stretch *a* before *b* 2 min after application of tetrodotoxin at a concentration of 1×10^{-8} g/ml Stretch monitored on lower trace Time bar 100 msec

min and no sign of conducted activity could be evoked regardless of the magnitude of the stretch used At lower concentrations of the toxin (*e g*, $1—2 \times 10^{-8}$ g/ml) the impulse response produced by a moderately strong stretch was usually eliminated within 10—15 min. By increasing the strength or velocity of the stretch, abortive spikes similar to those described by Katz (1950) could still be elicited for a varying duration of time

In general the action potentials generated during the static phase of the stretch were more susceptible to block by the toxin, this activity was abolished with toxin concentrations that left the dynamic response more or less unaffected This effect is illustrated by the records in Fig 1 In this experiment the spindle was subjected to a stretch with a duration of 300 msec This gave rise to a few dynamic spikes followed by a train of static impulses in the untreated preparation (Fig 1 *(a)*) It is noteworthy that the abolition of the static discharge produced by tetrodotoxin was not accompanied by any obvious reduction of the underlying sustained receptor potential (Fig 1 *(b)*)

B *Effects of tetrodotoxin on the action potential*

In order to follow the sequence of change of the conducted activity more closely a brief test stretch giving rise to a single spike was used in the following series of experiments

The immediate effect of the toxin was a gradual reduction in the amplitude and an increase in the time course of the regenerative response Using a test stretch whose amplitude was adjusted to twice that of the threshold for initiation of an action potential the minimum concentration of the toxin required to demonstrate the above mentioned effect varied from 2 to 5×10^{-9} g/ml With these concentrations only a partial suppression of the action potential was observed irrespective of the duration of the exposure to the toxin With a concentration of 1×10^{-7} g/ml the prolongation of the time course and the depression of the amplitude of the spike developed rapidly leading to an abolition of all afferent nerve activity within 2 min Before the abolition of the afferent nerve activity by tetrodotoxin the changes in the

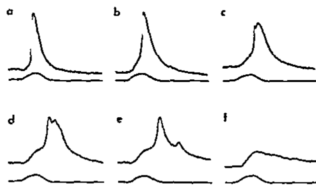


Fig 2 Decomposition and final blockage of action potential elicited by brief stretch *a* before, *b*—*f* at successive intervals of 2 min after application of tetrodotoxin at a concentration of 1×10^{-8} g/ml In *f*, only receptor potential is left Time bar 5 msec.

amplitude and the time course of the action potential were accompanied by a decomposition of the spike into two or three small components (Fig 2)

By washing with Ringer's solution the original spike height could usually be restored The time required for full recovery depended upon the concentration of the toxin and the duration of exposure Thus recovery was faster after short exposures than after prolonged ones it was also faster after treating the spindle with low concentrations than with higher ones For instance when the preparation was soaked in tetrodotoxin at a concentration of 4×10^{-8} g/ml for 2—3 min, recovery was usually complete in less than 10 min while it took 30—45 min after treatment with the same solution for 20—30 min

The diminution of the conducted activity was accompanied by several other marked effects As the amplitude of spike diminished the action potential generally underwent a decomposition into two or more small spikes (Fig 2), which gradually disappeared and left the pure receptor potential This decomposition of the spike was usually not seen when single strong test stretches were used The spike then appeared

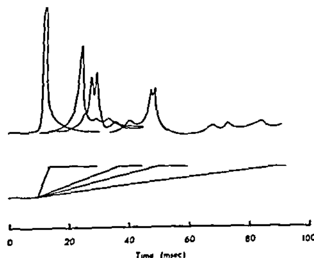


Fig 3 Decomposition of action potential by decrease of velocity of stretch Tracings of response obtained from spindle treated with tetrodotoxin at a concentration of 2×10^{-8} g/ml

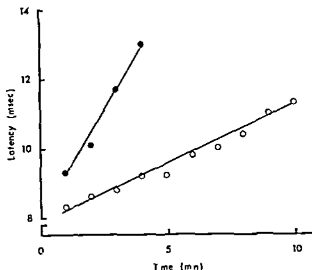


Fig 4 Increase in latency of action potential Spindle treated with tetrodotoxin in a concentration of 4×10^{8} g/ml (filled circles) and 1×10^{8} g/ml (open circles)

to diminish gradually in amplitude However, the effect of tetrodotoxin was revealed when the spindle was subjected to repeated stretches at frequencies of 5—10/sec The action potential then broke up into smaller spikes The same change occurred when the stretch was made slower In the experiment illustrated by the tracings in Fig 3 the spindle was treated with tetrodotoxin at a concentration of 2×10^{8} g/ml for 10 min A relatively strong stretch delivered thereafter elicited the initial dynamic spike only When the velocity of stretching was reduced the impulse became smaller and disintegrated into several smaller units

Simultaneously with these changes the latency of the action potential also increased In Fig 4 the latency of the spike is plotted against the duration of treatment with tetrodotoxin at two different concentrations Since the onset of the action potential could not be determined accurately the latency was measured as the interval between the initiation of stretch and the peak of the spike The increment in latency with time of exposure was greater with the higher concentration of toxin

After action potentials elicited by a weak or moderately large stretch had been abolished by a low concentration of the toxin the impulse could be made to reappear by increasing either the strength or the velocity of stretch The spike obtained in this way however was generally smaller than the original spike thereby indicating that there was a partial block of the sodium channels Fig 5 illustrates this effect The action potential elicited by a weak stretch was abolished by tetrodotoxin at a concentration of 1×10^{8} g/ml *(b)* When the strength of the stretch was increased a small spike appeared *(c)* With a further increase in the stretch the spike became larger *(d)* but never reached the same size as before blocking *(a)* In many experiments in which the muscle spindle was exposed to low concentrations of the toxin the amplitude of the spike appeared to be graded as a function of the strength of stretch A closer examination of this phenomenon revealed however that there was

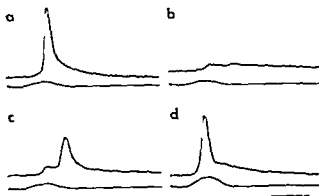


Fig. 5. Conducted activity evoked by increasing strength of stretch after blockage of threshold response. *a*, normal Ringer solution, action potential evoked by brief threshold stretch. *b*, blockage after application of tetrodotoxin in a concentration of 1×10^{-8} g/ml, *c*—*d*, responses evoked by slight increase of stretch. Time bar 5 msec.

not a continuous gradation in the spike amplitudes, but that the spike increased stepwise and the steps were not of equal amplitude.

These observations showed that the toxin caused a reduction in the sensitivity of the muscle spindle to stretch. This effect was also revealed by the gradually increasing threshold after application of the toxin. In the experiment illustrated in Fig. 6, the minimum stretch required to initiate a spike was measured during soaking of the spindle in tetrodotoxin at a concentration of 1×10^{-8} g/ml for 30 min. The minimal stretch at the end of this period was threefold greater than in the untreated preparation.

C. Effects of calcium on regenerative response and receptor potential

It has been suggested (Takata, Moore, Kao and Fuhrman, 1966) that inactivation of the propagated activity by tetrodotoxin might be influenced by changes in external calcium concentration. Experiments were performed to ascertain the interaction between calcium and tetrodotoxin. This was done by comparing the blocking action of tetrodotoxin in terms of the diminution of the spike or the time required to obtain

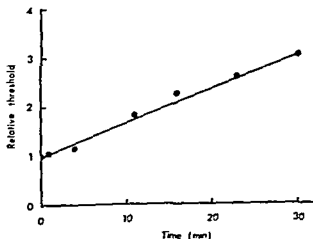


Fig. 6. Increase in threshold for evoking conducted activity in a spindle treated with tetrodotoxin in a concentration of 1×10^{-8} g/ml.

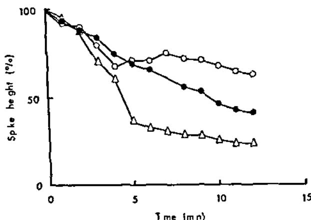


Fig 7 Effect of calcium on the blocking action of tetrodotoxin Decrease in amplitude of spike evoked by given test stretch after application of tetrodotoxin at a concentration of 2×10^{-8} g/ml in normal Ringer (filled circles), in Ringer with 5 times normal calcium concentration (open circles) and in Ringer without calcium and with EDTA (triangles) In each run the response was first observed for 5—10 min in the respective Ringer's solutions before tetrodotoxin was added Amplitude of spike just before application of tetrodotoxin given as 100 % After each run the preparation was kept in normal Ringer until the action potential had fully recovered

block at different concentrations of calcium in the bathing solutions Between each treatment the muscle spindle was allowed to recover completely in the normal Ringer's solution To exclude the possibility that the deterioration of the preparation might bias the results, the order in which the muscle spindle was exposed to the different calcium concentrations was varied from one experiment to another Prior to the application of tetrodotoxin, the spindle was allowed to remain for 10 min in the Ringer's solution with the calcium concentration to be tested This period of time proved to be sufficient for the establishment of a steady state

A fivefold increase in external calcium concentration produced a decrease in the blocking action of tetrodotoxin (2×10^{-8} g/ml) Contrariwise, the effectiveness of the toxin in blocking the conducted activity was enhanced when the external medium was depleted of calcium Representative findings are illustrated in Fig 7 In this experiment the time course of the reduction in amplitude of the spike after application of the toxin at a concentration of 2×10^{-8} g/ml was determined After recovery, the preparation was bathed with a calcium free solution plus EDTA for a period of 7 min With the reapplication of the same concentration of the toxin the impulse decreased rapidly in amplitude in the first 5 min and then declined more gradually The response remaining after 10 min largely represented the underlying receptor potential The effects of calcium could also be demonstrated by measuring the threshold changes as a function of the duration of treatment with tetrodotoxin The increase in threshold was slower when tetrodotoxin was applied in the presence of a high concentration of calcium than it was at normal calcium concentration

Earlier studies (Moore Blaustein Anderson and Narahashi 1967) indicated that the nerve blocking action of tetrodotoxin was not changed when sodium was replaced by lithium in the external solution Similar experiments in the present study confirm this observation

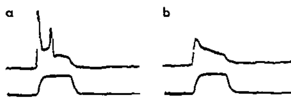


Fig. 8. Isolation of receptor potential after block of conducted activity with tetrodotoxin. Records of response to rapidly rising stretch with brief static phase before (*a*) and 5 min after (*b*) soaking the spindle in tetrodotoxin at a concentration of 2×10^{-8} g/ml. Time bar: 10 msec.

D. Effect of tetrodotoxin on the receptor potential

The exact size of the receptor potential could not be readily determined from recordings of the composite response, since it consisted of both the potential developed by the sensory endings and the conducted spikes of the afferent fiber. Therefore it was difficult to know whether tetrodotoxin also caused a reduction of the generator potential in addition to blocking the conducted activity. By critical adjustment of the parameters of the stretch, however, a reliable evaluation of the size of the receptor potential could be made before treatment of the muscle spindle with tetrodotoxin. Fig. 8 shows records of impulse and generator potentials obtained with a relatively strong stretch. This stretch gave rise to two dynamic spikes and was adjusted to terminate just before the appearance of the first static spike. The steady potential developed following the dynamic phase may therefore be regarded primarily as the static phase of the receptor potential. It can be seen in record *b* (and in Fig. 1) that the level of the steady potential was unchanged after blocking of the impulse activity with tetrodotoxin. A similar analysis was carried out with respect to the action of tetrodotoxin on the dynamic part of the receptor potential. The results of these experiments were consistent with those on the static phase, thereby showing that there was very little, if any, effect upon the receptor potential.

To eliminate the possibility that the persistence of the receptor potential was due simply to minor differences in the sensitivity of the sensory endings to the drug, the effects of high concentrations were examined. Record *a* in Fig. 9 shows the responses obtained after blocking the conducted activity with 2×10^{-8} g/ml. The concentration was then increased to 0.1 and 1.0×10^{-6} g/ml in records *b* and *c* respectively, and the spindle was soaked in each of these solutions for a period of 20 min. A close inspection of the records shows that the receptor potential was not affected by the toxin in these concentrations, which were from 5 to 50 times greater than those required to block the conducted activity.

The observation that the receptor response remained unchanged after blocking the



Fig. 9. Absence of effect of tetrodotoxin on the receptor potential. Records of receptor potential after treatment of spindle for 20 min with tetrodotoxin at a concentration of 2×10^{-8} g/ml (*a*), 1×10^{-7} g/ml (*b*) and 1×10^{-6} g/ml (*c*). Time bar: 10 msec.

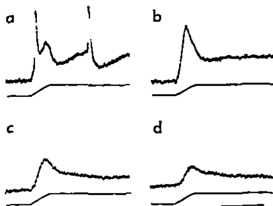


Fig 10 Effect of removal of sodium *(c)* and thereafter also of calcium *(d)* on receptor potential *(b)* obtained after blocking of impulse activity *(a)* with tetrodotoxin at a concentration of 1×10^{-7} g/ml Time bar 30 msec

conducted activity suggests that the sodium carrying system of the receptor membrane was not affected by the toxin Further evidence for this was obtained from experiments in which sodium was removed from the bath solution after the conducted activity was blocked with tetrodotoxin When choline chloride was substituted for sodium, the concentration of tetrodotoxin being unchanged, there was a reduction of approximately 50 % of the receptor potential (Fig 10) The effect was reversible and when the sodium concentration subsequently was restored to its normal value, the receptor potential returned to its original height within 10—15 min in the presence of tetrodotoxin

Replacement of sodium by lithium caused no detectable changes in the characteristics of the action potential or receptor potentials of the muscle spindle These findings indicated that tetrodotoxin does not affect the passage of lithium through the receptor membrane during excitation, although the ionic passive transport associated with the action potential is blocked When lithium was replaced by choline chloride the receptor potential was reduced by the same amount as when sodium was removed

The persistence of the receptor potential in the absence of sodium raises the question about what ions are responsible for the generation of the receptor potential It can be postulated that a small amount of sodium ions may still remain in the space surrounding the sensory endings An alternative but not mutually exclusive hypothesis is that calcium may be involved in generating the receptor potential To test the latter hypothesis calcium was removed from the external solution after exposing the spindle to a sodium free solution with tetrodotoxin (1.0×10^{-7} g/ml) for 20 min This was found to cause a fall of the receptor potential by approximately 20 % (Fig 10 *d*) Therefore it appears that calcium ions are responsible directly or indirectly for the development of part of the receptor potential

Discussion

The present results demonstrate that tetrodotoxin blocks the conducted activity of the frog muscle spindle without producing any significant action on the receptor potential The analysis of the blocking action of the toxin showed that the block of the

conducted activity was preceded by a series of changes involving an increase in threshold of the nerve a reduction in amplitude of the spike and a decomposition of the conducted impulse into smaller spikes An increase in the concentration of external calcium reduced and delayed the blocking action of tetrodotoxin while removal of this ion had the opposite effect.

The changes in threshold and spike amplitude by tetrodotoxin are consistent with earlier observations that tetrodotoxin blocks the early transient sodium current The decomposition of the action potential of the muscle spindle afferent nerve fiber into subcomponents is less easy to interpret on the basis of earlier findings (Nakahashi *et al* 1960 Narahashi Moore and Scott 1964, Nakamura *et al* 1965, Takada *et al* 1966 Kao 1966) The structural organization of the spindle may furnish a possible explanation for this effect of the toxin The muscle spindle in the frog is innervated by a single afferent fiber which divides into a system of branches of myelinated fibers (Karlsson Andersson Cedergren and Ottoson 1966) These branches ultimately terminate in sensory chains The receptor potential produced in these chains spreads into the myelinated branches where impulses are initiated and propagated toward the stem fiber Because of the short length of these branches the entire system tends to be active in synchrony under normal conditions although the activity of the individual branches can be distinguished in detailed analysis (Katz 1950 Ottoson and Shepherd 1968) Tetrodotoxin seems to act by producing blockage in the branching tree of nerve terminals within the spindle The time course of development of block depends on the concentration of the toxin and its rate of diffusion into the different intracapsular compartment (*cf* Karlsson *et al* 1966) It is likely that the decomposition of the action potential into smaller units may be attributed to differences in blocking action of the toxin in different regions of the spindle leading to a desynchronization Impulses in individual branches would arrive at the first node of the stem fiber asynchronously and would appear in the recording as small abortive spikes. Further evidence for this interpretation is based on the observation that different branches of an untreated spindle can be activated separately by certain modes of stimulation

The analysis of the response before and during the blocking of the action potential produced by tetrodotoxin showed that there was no significant effect on the static phase of the receptor potential The effect on the dynamic component was less readily determined since the magnitude of this part of the response could not be accurately measured in the composite response before blocking Indirect evidence suggests however that tetrodotoxin does not affect the dynamic potential either Furthermore it would appear unlikely that the toxin would act on one phase and not on the other since the static and the dynamic phases of the potential are produced by the same membrane

Removal of sodium was accompanied by a reduction of the receptor potential of the muscle spindle The reduction in the receptor potential in this study corresponded to the expected value under the assumption that tetrodotoxin did not block the sodium channels These observations indicate that the toxin is unable to affect the

sodium channels involved in the generation of the receptor potential of the muscle spindle

We suggest that the sodium carrying system of the receptor membrane is different from that of the membrane producing the propagated action potential Tetrodotoxin, it appears, does not have access to the gates controlling the flow of sodium ions across the sensory membrane of the spindle

An increase in external calcium concentration effectively retarded the action of tetrodotoxin on the propagated activity of the muscle spindle A similar observation on the interaction between calcium and the action of tetrodotoxin has been made on the giant axons of the squid (Takada *et al* 1966) Thus it seems feasible that the action of tetrodotoxin on sodium current is directly linked with the gate controlling properties of calcium (Tobias, Agin and Pawlowski 1962 Lettvin *et al* 1964, Albuquerque and Thesleff 1968)

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Effects of Bicarbonate and Thiocyanate on Fluxes of Na and K, and on Glucose Metabolism of Actively Transporting Human Red Cells

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Abstract

WIETH, J O *Effects of bicarbonate and thiocyanate on fluxes of Na and K, and on glucose metabolism of actively transporting human red cells* Acta physiol scand 1969 *75* 313—329

It has been shown in a previous work that sodium influx is trebled, whereas potassium efflux is slightly reduced when erythrocytes are suspended in a 142 mM bicarbonate medium containing ouabain The present work deals with the active transport of sodium and potassium occurring in the presence of 142 mM bicarbonate (38° C, pH 7 40) Sodium influx and potassium efflux were unaffected by the removal of ouabain, whereas sodium efflux, potassium influx and glucose metabolism were all strongly stimulated The relation between ouabain sensitive ion transport and the calculated extra formation of ATP was 3 1 moles Na/mole ATP and 2 3 moles K/mole ATP, in agreement with values previously found on red cells transporting Na and K in plasma Red cells incubated in the bicarbonate medium accumulated potassium until extracellular potassium concentration had been reduced to 1 8—2 mM The normal volume regulation of the cells was severely interfered with because accumulation of sodium and potassium was accompanied by a net flux of water of 6 g per meq cation All [illegible] to the increased sodium influx leading [illegible] which in turn stimulated active cation [illegible] rn over The properties of the ouabain [illegible] he presence of bicarbonate

Both potassium efflux and sodium influx are increased by 200—300 per cent in the presence of 120 mM thiocyanate It was found, however, that active cation transport is not stimulated in a 120 mM thiocyanate medium On the contrary the ouabain sensitive ion transport was steadily decreasing during incubation in spite of conditions otherwise favouring transport of Na and K i e increasing extracellular potassium and intracellular sodium concentrations

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In a previous study (Funder and Wieth 1967 b) it was shown that the cation permeability of human red cells was altered, when chloride ions in the external medium were substituted by other monovalent anions The changes were particularly pronounced when chloride was replaced by thiocyanate or bicarbonate sodium influx into ouabain treated cells was trebled in the presence of 142 mM bicarbonate, whereas potassium efflux was slightly reduced, in the presence of 120 mM thiocyanate both sodium influx and potassium efflux increased by 200—300 per cent

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In the present work the effect of bicarbonate and of thiocyanate on sodium and potassium fluxes has been studied in the absence of ouabain to establish (a) whether the so-called passive fluxes (Na influx and K-efflux) are influenced by ouabain, and (b) how the ouabain sensitive transport mechanism responds to bicarbonate or thiocyanate in the absence of cardiac glycosides. Sodium influx and potassium efflux were unaffected by the removal of ouabain. In the bicarbonate medium both active transport of sodium and potassium and the rate of glycolysis were strongly stimulated, whereas active transport was inhibited progressively with time when erythrocytes were incubated in a NaSCN containing medium.

Methods

Sampling, washing, and incubation of red cells at 38° C and pH 7.40 were carried out according to the techniques previously described (Funder and Wieth 1967 a and b). The same references contain information about analyses employed, the determination of net fluxes of sodium, potassium and water, and about the simultaneous determination of unidirectional Na and K fluxes by means of ^{22}Na and ^{42}K. The fractional cell volume in the incubate was 0.2—0.4. The isolation of red cells for chemical and radioactive analyses was carried out at 0° C, except in 3 of the experiments, in which the determination of ^{36}Cl distribution between [illegible]

[illegible] In two of the experiments carried out in the [illegible] was added after 5—8 hrs in order to provide sufficient substrate for glycolysis. In these experiments it was observed that the rate of glycolysis was independent of glucose concentrations within the range of 2—10 mM. In some experiments (see Fig. 2) the potassium concentration was varied between 0 and 20 mM by adding the appropriate amounts of $KHCO_3$ to a potassium free bicarbonate medium. In a few other experiments (see Fig. 3) the bicarbonate medium was modified by substituting sodium with choline (choline bicarbonate purum Fluka, Switzerland).

Types of experiments. One series of 8 expts. was carried out in the sodium bicarbonate medium. The results obtained during the first 3 hrs of incubation following equilibration were compared with results previously obtained in the presence of ouabain (Funder and Wieth 1967 b). This comparison is shown in Table II, III and V. Four of the experiments were continued for 6—22.5 hrs. Typical results from a long lasting experiment are presented in [illegible]

[illegible]

The distribution of radioactive chloride between cells and bicarbonate medium was determined in 3 of the experiments. ^{36}Cl was obtained as HCl with a specific activity of 0.5 mC/mmole. The HCl was titrated with sufficient NaOH to make a neutral solution of ^{36}Cl labelled NaCl. The isotope was added to the cell suspension 10 minutes before starting the experiment by the addition of ^{42}K and ^{22}Na. The amount of radioactive chloride employed was 0.1 μC/ml cell suspension corresponding to a chloride concentration of 0.2 meq per litre. Counting of radiation from ^{42}K and ^{22}Na followed the technique of Funder and Wieth (1967 b). As only γ radiation with energies above 1 Mev was measured the measured activities of potassium and sodium were not influenced by the presence of ^{36}Cl. After a week when control countings showed that the activity of ^{42}K had decayed ^{36}Cl was counted in a Tricarb *liquid scintillation spectrometer* (Model 3324). After precipitating approximately 150 mg of erythrocytes or 200 mg of medium with 1 ml of 0.6 M perchloric acid 50 or 100 μl of the supernatant were transferred to counting vials containing 10 ml of the scintillation medium (toluene 70 per cent (v/v), ethanol 30 per cent (v/v), 2.5-diphenyloxazole 3.5 g/litre, 1.4 bis-2-(4-ethyl-5-phenyl-oxazolyl)benzene 0.07 g/litre). The presence of other electrolytes did not interfere with the counting of ^{36}Cl. The recovery of ^{36}Cl added to

electrolyte media was 100 3 per cent (coefficient of variation 1 6, n=12) The highest preci sion in the separation of the β radiations from ^{22}Na and ^{36}Cl (having maximal energies of 0 54 and 0 71 Mev respectively) could not be obtained by β spectrometry, but was achieved when the sum of β radiation in the energy range from 0 1 to 0 8 Mev was corrected for the activity originating from ^{22}Na determined by means of its γ radiation By this method the mean recovery of ^{36}Cl in the presence of ^{22}Na in the concentrations found in the experiments was found to be 100 3 per cent (coefficient of variation 0 38, n=6) Repeated determinations of the counting rates of a sample of ^{22}Na showed that the ratio β radiation registered/γ radiation registered stayed within the range 2 70$\pm$0 03 the counting efficiency of the γ radiation of ^{22}Na being 30 per cent and that of the β radiation being 81 per cent

In order to establish whether the results obtained with ^{36}Cl were comparable with results ve determined the distribution of plasma pH in the pH range be ratio (r Cl=^{36}Cl activity per kg 3 17–0 34 pH_{plasma} (r=0 999) The extreme values of the distribution ratio 0 91 and 0 57 agreed reasonably with the values of 0 93 and 0 58 calculated from the regression line 3 319—0 359 pH_{plasma} as found by chemical analysis of chloride (Funder and Wieth 1966 a)

A series of experiments was carried out to determine the influence of extracellular potassium and of intracellular sodium concentration on potassium influx in the presence of bi carbonate (Fig 2 and 3) In order to minimize changes of intracellular sodium concentra tion during the incubation period, red cells were only incubated for 20 min in the experiments of Fig 2 and in those of Fig 3 in which intracellular sodium concentration was below 30 meq/kg water In such short lasting experiments potassium influx (M_n) was calculated from the initial increase of intracellular radioactivity

$$M_n = \frac{\text{cpm/kg cell solids}}{\text{cpm/meq extracellular K}} \cdot \frac{60}{20} \text{ (meq/kg cell solids hr)}$$

To obtain red cells with intracellular Na concentrations varying between 3—100 mM it was necessary to pretreat the cells In the choice of pretreatments we aimed at a certain degree of overlapping between the sodium concentrations of the cells so that specific effects of the pretreatment on the transporting capacity of the cells could be disclosed Four methods were employed to modify the intracellular Na and K concentrations of red cells Sodium depleted cells were obtained in one of two ways (a) by incubating the cells 1 to 6 hrs in the choline bicarbonate medium containing 20 mM potassium at 38° C and pH 7 40 (b) In other experiments the cells were incubated 2—4 hrs at 0° C in a 0 15 M KSCN medium In this medium the cells lose sodium due to the pronounced increase of sodium permeability previously observed at 0° C in the presence of thiocyanate (Funder and Wieth 1967 b) Later investiga tions have shown that the potassium permeability is similarly increased by SCN at 0° C so that the cells exchange almost equimolar amounts of Na and K Incubation of cells at 0° C in the presence of thiocyanate is well suited for rapid modification of intracellular sodium and potassium contents because the permeability changes are completely reversible on removal of thiocyanate by washing the cells in a medium containing another anion Loading of cells with sodium was performed in one of two ways (a) in 8 experiments of Fig 3 cells were stored from 5—50 hrs at 0° C in the NaSCN medium specified above The cells maintain their transport capacity after thiocyanate incubation (*cf* Fig 3) whereas the rate of active cation transport has been reported to decrease by 1/3 following prolonged incubation (63 days) at 2° C in a sodium chloride containing medium (Post *et al* 1960) (b) In 7 of the experi ments of Fig 3 a moderate increase of intracellular sodium concentration was obtained by in cubating red cells in plasma at 0° C from 6 to 36 hrs After pretreatment the cells were washed 3—5 times in choline bicarbonate medium and incubated in the medium specified in the legend of the figure

Fluxes of sodium and potassium in the presence of thiocyanate (Table VI and VII) were determined according to the technique of Funder and Wieth (1967 b) the only exception be ing that ouabain was omitted from the electrolyte medium

Results

I Sodium and potassium transport in the presence of a high bicarbonate concentra tion

The results from a typical experiment are shown in Table I Accumulation of both sodium and potassium was observed when red cells were incubated in

TABLE I Cellular concentrations and net fluxes of sodium, potassium, and water of red cells suspended for 9 hrs in the 142 mM $NaHCO_3$ medium specified in the method section (38° C, pH 7 40, donor J F) The distribution of ^{36}Cl between cells and medium and the extracellular potassium concentration is shown in the lower part of the table Further explanation is found in the text

hr	0	1 5	3	4	6	7	8	9	net flux 0—9
Cell Na									
(meq/kg cell water)	13 1	17 9	21 3	23 6	28 1	30 0	32 5	33 0	
(meq/kg cell solids)	25 9	36 5	45 6	51 6	64 7	70 2	77 6	80 4	54 5
Labelled cell Na									
(meq/kg cell solids)	0	24 4	39 6	47 6	61 5	71 1	77 0	83 2	
Cell K									
(meq/kg cell water)	128 3	127 1	123 1	122 0	117 3	115 3	113 3	111 3	
(meq/kg cell solids)	253 5	259 2	264 0	266 5	269 8	270 4	270 9	271 1	17 6
Cell (Na + K)									
(meq/kg cell water)	141 4	145 0	144 4	145 6	145 4	145 3	145 8	144 3	
(meq/kg cell solids)	279 4	295 7	309 6	318 1	334 5	340 6	348 5	351 5	72 1
Cell water									
(g/kg cell solids)	1976	2040	2145	2185	2300	2345	2390	2436	460
Chloride distribution									
^{36}Cl (cell water) / ^{36}Cl (medium)	0 715	0 714	0 669	0 666	0 746	0 733	0 753	0 689	
Extracellular K									
(meq/l)	4 5	3 6	2 9	2 5	2 0	1 94	1 88	1 85	

medium containing 142 mM sodium bicarbonate Due to the persistence of sodium extrusion in the absence of ouabain, the rate of sodium accumulation was only 50 per cent of the rate previously found in the presence of ouabain, *cf* Table II In the absence of cardiac glycosides there was also an accumulation of potassium, 17 6 meq per kg cell solids during 9 hrs The accumulation of potassium decreased with the falling extracellular concentration Potassium net flux was 10 5 meq per kg cell solids during the first 3 hrs of the experiment whereas the net flux was only 1 3 meq between the 6th and the 9th hour The uptake of cations was accompanied by a net transfer of water to the cells of 460 g water per kg cell solids When the net flux of (Na+K) was correlated to water uptake the cells took up 460/72 = 6 4 g water per meq cation It can be seen from the constancy of the sum of (Na+K) in the cell water (143 ± 2 meq/kg cell water) that the contribution of these cations to the osmolality of the intracellular fluid was constant throughout the experiment

TABLE II Comparison of the net fluxes of sodium, potassium and water occuring in human erythrocytes suspended in the 142 mM bicarbonate medium with and without ouabain All experiments were carried out at 38° C pH 7 40 The results obtained in the presence of ouabain were taken from Funder and Wieth (1967 b) $k_1(hr^{-1})$ is the rate constant of sodium influx Influx = k_1 (C_o), C_o being the extracellular sodium concentration S E is stated in parenthesis

		142 mM bicarbonate medium (ouabain 0 M)	142 mM bicarbonate medium (ouabain 3 10⁻⁴ M)
Number of experiments		8	5
Cell sodium (meq/kg solids)	0 hr	25 3 (2 2)	28 6 (2 4)
	3 hrs	45 4 (2 2)	68 1 (1 6)
Sodium net flux (meq/kg solids 3 hrs)		20 1 (1 3)	39 5 (1 5)
Sodium influx k_1 (hr^{-1})		0 142 (0 007)	0 144 (0 004)
Extracellular potassium (meq/l)	0 hr	4 7 (0 4)	5 6 (0 6)
	3 hrs	3 3 (0 3)	8 1 (0 6)
Cell potassium (meq/kg solids)	0 hr	255 4 (4 1)	252 2 (5 5)
	3 hrs	263 4 (4 2)	241 5 (5 7)
Potassium net flux (meq/kg solids 3 hrs)		7 6 (0 9)	— 10 7 (0 4)
Cell water (g/kg solids)	0 hr	1943 (29)	1932 (37)
	3 hrs	2101 (38)	2109 (29)
Water net flux (g/kg solids 3 hrs)		158 (13)	187 (7)
Sodium + potassium net flux (meq/kg sol ds 3 hrs)		27 8 (1 0)	28 8 (1 8
Water net flux per meq Na — K accumulated (g meq)		5 7 (0 5)	6 5 (0 6)

The distribution ratio of ^{36}Cl between cell water and medium was between 0 67—0 75 during the whole experiment A similar constancy of ^{36}Cl distribution was found in two other experiments of 3 and 8 hrs duration

The results of the experiment shown in Table I were confirmed in 8 expts summarized in Table II For comparison the results previously obtained in the presence

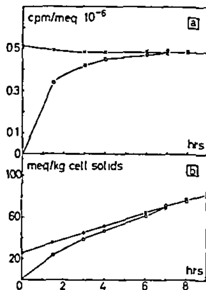


Fig 1 a) The time course of extracellular (×—×) and of intracellular specific activity (○—○) of ^{22}Na during 9 hrs' incubation of red cells in the bicarbonate medium The results are from the experiment shown in Table I

b) The time course of total cellular sodium content determined by flame photometry (●—●), and of the content of labeled sodium calculated from the activity of cells and the specific activity of extracellular sodium (○—○) Total exchange of intracellular sodium was accomplished in 6—7 hrs

of ouabain are shown in the right hand column of the table The average initial contents of sodium, potassium, and water were identical in the two series During 3 hrs incubation at 38° C sodium net flux was 39 meq/kg solids in the presence of ouabain, but only 20 meq when Na extrusion was not inhibited The potassium loss from ouabain treated cells was contrasted by a net uptake of 7 6 meq/kg cell solids in the transporting cells The net fluxes of (Na+K) and the amount of water transferred per meq of cation were of the same magnitude in the two series The volume of the red cells thus increased to the same degree whether ouabain was present or not, because the active accumulation of potassium in the transporting cells sufficed to compensate the smaller net flux of sodium Sodium influx was not at all affected by ouabain, as indicated by the rate constants of sodium influx (k_1 0 144 and 0 142 corresponding to an influx of 20 5 meq per kg solids and hour at an extracellular sodium concentration of 142 mM) The effect of ouabain on sodium efflux will be dealt with in section 3 Complete exchange of intracellular sodium was accomplished in 4—7 hrs when the cells transported Na actively Fig 1 a shows the time course of the specific activities of extra and intracellular sodium, and Fig 1 b shows the accumulation of total and of labelled sodium during an experiment of 9 hrs duration. The results shown in Fig 1 are representative of the experimental series After 3 hrs incubation the ratio between the specific activities of intracellular and extracellular sodium showed a mean value of 0 84 (S E 0 03, n=7) After an incubation period of 6 hrs or more the mean fractional exchange of intracellular sodium was 0 984 (S E 0 014 8 samples from 4 expts)

The unidirectional potassium fluxes were determined in 4 expts (Table III) Like sodium influx potassium efflux was the same whether ouabain was present or not. The average value of 12 6 meq/kg cell solids per 3 hrs (Table III) is close to the value of 11 7 meq previously found in the presence of ouabain (Funder and Wieth

TABLE III Unidirectional potassium fluxes of red cells suspended in the 142 mM bicarbonate medium ^{42}K was added to the cell suspension at the start of the experiment, and the potassium efflux was calculated as the difference between potassium influx and net flux

Experiment	Extracellular potassium (meq/l)		Intracellular sodium (meq/kg water)		Potassium fluxes (meq/kg cell solids 3 hrs)		
	0 hr	3 hrs	0 hr	3 hrs	Influx	Efflux	Net flux
5 6 65	4 8	3 6	10 6	18 4	21 8	14 4	7 4
6 7 65	4 1	3 1	13 6	19 5	20 5	12 1	8 4
3 7 66	4 0	2 7	19 4	27 5	18 8	12 2	6 6
6 8 67	4 5	2 9	13 1	21 3	21 6	11 2	10 4

1967 b) *In the same work we found that potassium influx in the presence of ouabain* is very small, when cells were suspended in the bicarbonate medium (0 3 meq/kg cell solids hr at an extracellular potassium concentration of 6 8 meq/litre) Almost the whole potassium influx in the experiments shown in Table III must therefore have been mediated by the active, ouabain sensitive transport mechanism Potassium influx remained between 5—8 meq/kg solids hour in all subperiods of these 4 expts, in spite of the fact that extracellular potassium concentration was steadily decreasing

The rate of hemolysis was slow during the first 9 hours of incubation in spite of the progressing swelling of the cells (hemolysis increasing from 0 02 to 0 1 per cent of cell hemoglobin liberated per hour) When the incubation was continued, hemolysis increased In one experiment 9 3 per cent of total hemoglobin had been liberated from the cells after 22 5 hrs incubation The water content of the surviving cells was 2593 g per kg cell solids, and the intracellular sodium concentration was 79 5 meq/kg cell water The ultimate fate of erythrocytes which are incubated in the bicarbonate medium is that of osmotic hemolysis *The cells are bound to accumulate cations and* swell The relation between accumulation of sodium and potassium will be determined by the extracellular potassium concentration When external potassium is low, the cells will accumulate sodium at a high rate When osmotic hemolysis commences, Na extrusion from the surviving cells will be stimulated by the potassium liberated, but the rate of swelling is not diminished because the decreased net flux of sodium is compensated by an increased influx of potassium

2 The effects of extracellular potassium and of intracellular sodium on the rate of potassium transport

The ouabain sensitive sodium and potassium transport is stimulated when extracellular potassium or intracellular sodium concentration is increased (Glynn 1956 Post and Jolly 1958) The experimental conditions in the experiments shown in Table I and II are therefore complex because the increasing intracellular sodium concentration may be expected to counteract the effect of the falling extracellular

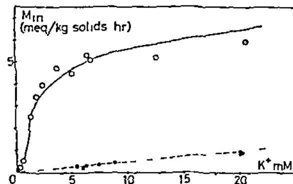


Fig 2 The relation between extracellular potassium concentration and potassium influx (M_{in}) of human erythrocytes suspended in media containing 142 mM $NaHCO_3$ (*cf* method section) The full line represents the potassium influx found by Sachs and Welt (1967) on red cells suspended in media containing 142 mM chloride (further explanation in text) The broken line in the lower part of the figure shows the potassium influx found in the presence of ouabain 3 10^{-4} M $M_{in} = 0\ 047$ K_{medium} (Funder and Wieth 1967 b) Five of the ouabain experiments were taken from the work cited and 3 additional experiments were performed at an extracellular potassium *concentration of 20 mM The intracellular sodium concentration was in all experiments within* the range 11 2—13 4 meq/kg cell water (Donor J W pH 7 40 38° C)

potassium concentration In order to establish whether or not the sensitivity of the transport system to the above mentioned stimuli is altered when chloride is replaced by bicarbonate, potassium influx was determined as a function of extracellular potassium concentration at a fixed level of intracellular sodium and as a function of intracellular sodium concentration at a fixed level of extracellular potassium (Fig 2 and 3) It appears from Fig 2 that the potassium influx was highly dependent on the extracellular potassium level at concentrations below 5 meq/litre The results of Fig 2 may be compared with those of Sachs and Welt (1967) who employed a bicarbonate free electrolyte medium containing 142 mM chloride Sachs and Welt found that the curve relating potassium influx to extracellular potassium concentration was S shaped and could be adequately described by an equation of the type

$$M_{in} = \frac{a}{1 + \frac{b}{(K)} + \frac{c}{(K)^2}} + (d\ K) \ \text{(meq/kg solids hr)}, \qquad (1)$$

where a b c and d are constants and K is the extracellular potassium concentration The full line in Fig 2 is a graph of equation (1), using values of 6 1 6 and 0 6 for the constants a b and c These values are identical with those found by Sachs and Welt (1967) if the reasonable assumption is made that the water content of the red cells employed in their study was about 680 g/l The value of 0 047 hr^{-1} employed for the constant d is the mean value of the rate constant of potassium influx into ouabain treated cells (Funder and Wieth 1967 b) The broken line in the lower part of Fig 2 indicates the potassium influx found in the presence of ouabain The mean intracellular sodium concentrations during the experiments of Fig 2 are shown in Table IV, which also shows the sodium influx found in these experiments Although the experiments were of only 20 min duration the intracellular sodium concentration rose, and the mean concentration was 2 4 meq above the normal value of 10 meq/kg cell water (Funder and Wieth 1966 b) However sodium concentration was also a little above normal in the experiments reported by Sachs and Welt (1967), so it seems justified to conclude from the approximate qualitative and quan

TABLE IV Cellular sodium concentration, extracellular potassium concentration and influxes of sodium and potassium into red cells suspended in the 142 mM $NaHCO_3$ medium (38° C, pH 7 40, donor J W) The results are derived from the same expts as shown in Fig 2

Cell sodium (meq/kg cell water)	Extracellular sodium (meq/l)	Extracellular potassium (meq/l)	Sodium influx (meq/kg cell solids hr)	Potassium influx
11 2	142	0 35	18 2	0 35
13 4	142	0 65	22 6	1 02
12 7	142	1 3	21 0	2 53
12 9	142	1 8	20 4	3 41
12 0	142	2 3	19 7	3 96
12 2	142	3 6	20 3	4 74
12 9	142	4 9	19 9	4 47
12 6	142	6 4	18 5	5 31
11 8	142	6 6	18 2	5 16
11 8	142	12 5	17 9	5 23
13 0	142	20 4	20 0	5 99

titative agreement between the results of the two investigations that the properties of the ouabain sensitive potassium influx does not change, when chloride is replaced by bicarbonate

Fig 3 shows that the potassium influx at a constant extracellular potassium concentration of 20 meq/l was increasing with intracellular sodium concentration The S shape of the curve is even more evident than in Fig 2 Half maximal saturation of the ouabain sensitive potassium influx was obtained at an intracellular sodium concentration of 15 meq/kg cell water At intracellular sodium concentrations between 75—100 meq/kg cell water the total potassium influx was 11—12 meq/kg cell solids per hour, twice the maximal rate found at an intracellular sodium concentration of 12—13 meq/kg cell water (Fig 2) The broken line in the lower part of Fig 3 shows that potassium influx was unaffected by the intracellular sodium concentrations when ouabain was present in the medium

3 Relation between the rates of sodium and potassium transport and the rate of glycolysis

The rate of glycolysis has previously been determined in red cells suspended in the 142 mM bicarbonate medium in the presence of ouabain In Table V these data are compared with the rate of glycolysis found during the first three hours in the 8 expts performed with transporting cells (viz Table II) When the red cells were allowed to transport sodium and potassium glucose consumption increased by 1 4 and lactate production by 2 8 mmoles/kg cell solids per hour Because the turnover of one mole of glucose was accompanied by the formation of 1 92—1 97 moles of lactate we believe that the concentration of glycolytic intermediates were in a steady state in the

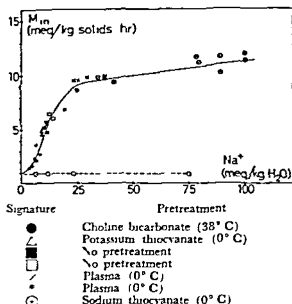


Fig 3 The relation between intracellular sodium concentration and potassium influx (M_{in}) of human erythrocytes suspended in bicarbonate media containing 20 mM potassium (donor J W., pH 7 40, 38° C) The signatures employed in the figure refer as follows to the pretreatment and the incubation media employed (*cf* method section)

Signature	Pretreatment	Incubation medium
●	Choline bicarbonate (38° C)	choline bicarbonate
∠	Potassium thiocyanate (0° C)	choline bicarbonate
■	No pretreatment	choline bicarbonate
□	No pretreatment	sodium bicarbonate
/	Plasma (0° C)	choline bicarbonate
*	Plasma (0° C)	sodium bicarbonate
⊙	Sodium thiocyanate (0° C)	sodium bicarbonate

The broken line in the lower part of the figure (○—○) shows the potassium influx found in the presence of ouabain (3 10^{-4} M)

two experimental series The difference in lactate production of 2 8 mmoles/kg cell solids per hour was therefore assumed to signify an extra ATP production in the transporting cells of 2 8 mmoles ATP

To be able to evaluate the relation between energy metabolism and the amounts of sodium and potassium transported by an ouabain sensitive mechanism, the magnitudes of ouabain sensitive sodium efflux and potassium influx must be known. Unidirectional sodium fluxes were measured in all 8 expts (Table II) Total sodium influx during 3 hrs was 0 142 142 6 3 − 60 75 meq/kg cell solids Because the net flux of sodium was almost linear in time (Fig 1 b) the ouabain insensitive sodium efflux could be calculated as 0 14 35 35 3 = 14 85 meq per kg cell solids per 3 hours where 0 14 is the rate constant of sodium efflux in the presence of ouabain (Funder and Wieth 1967 b and 35 35 is the mean value of intracellular sodium content (Table II) Net flux of sodium was 20 1 meq per kg cell solids during 3 hrs so the amount of sodium extruded by the ouabain sensitive transport mechanism was 60 75 − (14 85 + 20 1) = 25 8 meq/kg cell solids per 3 hrs or an average of 8 6 meq per hour The stoichiometrical relation of sodium transport to the extra ATP formation was thus 8 6/2 8 = 3 07 close to the value found when erythrocytes with a normal low sodium content transporting sodium at a rate of 5 meq/kg cell solids · hour were incubated in plasma (Funder and Wieth 1967 a)

Unidirectional potassium fluxes were only determined in 4 of the experiments but these experiments rendered evidence that potassium efflux is not affected by the presence of ouabain (Table III) The ouabain sensitive potassium influx could therefore be calculated in a way similar to that employed in the calculation of sodium

TABLE V Comparison of the glucose consumption and lactate production of human erythrocyte suspended in the 142 mM bicarbonate medium with and without ouabain All results were from the first 3 hrs following equilibration in the bicarbonate medium The result obtained in the presence of ouabain were taken from Funder and Wieth (1967 b)

	Glucose consumption (mmole/kg cell solids hr)	Lactate production (mmole/kg cell solids hr)	Lactate / Glucose
142 mM bicarbonate medium (ouabain 0 M)	4 66	9 15	1 97
S E	0 20	0 46	0 08
Number of experiments	8	8	8
142 mM b carbonate medium (ouabain 3 10 $^{-4}$ M)	3 30	6 34	1 92
S E	0 19	0 44	0 08
Number of experiments	5	5	5
Difference	1 36	2 81	
(Students t test) P	< 0 001	< 0 005	

efflux The ouabain insensitive potassium influx is 0 2 meq/kg cell solids hr at an extracellular K concentration of 4 meq/litre (Funder and Wieth 1967 b) In the same work potassium efflux in the presence of ouabain was found to be 3 9 meq/kg cell solids hr in agreement with the value of 4 2 meq found at slightly higher intracellular potassium concentrations in the absence of ouabain (Table III) The average net flux of potassium in the 8 expts shown in Table II was 2 5 meq/hr so that potassium influx averaged 2 5+4 2=6 7 meq/kg cell solids hr Subtracting the ouabain insensitive influx of 0 2 meq/hr the amount of potassium transported per extra mmole of ATP produced was 6 5/2 8=2 3 meq per mmole close to the value of 2 5 previously found for normal cells incubated in plasma

4 Sodium and potassium transport in the presence of a high thiocyanate concentration

Potassium efflux and sodium influx have previously been shown to be increased by 250—300 per cent in the presence of 120 mM thiocyanate (Funder and Wieth 1967 b) Since active transport is stimulated by extracellular potassium and by intracellular sodium the thiocyanate medium might be expected to provide favourable conditions for high rates of Na and K transport That this was not the case is demonstrated in Table VI in which results obtained in the absence of ouabain are compared to those previously found in the presence of this drug Net fluxes of sodium and potassium were only reduced by some 30 per cent in the absence of ouabain in spite of the accumulation of intracellular sodium and of extracellular potassium Sodium influx and potassium efflux were not affected by the presence of oual

TABLE VI Comparison of fluxes of sodium, potassium, and water of human erythrocytes suspended in the sodium thiocyanate medium without and with ouabain $3 \cdot 10^{-4}$ M (38° C, pH 7.40). The results obtained in the presence of ouabain were taken from Funder and Wieth (1967 b)

		Ouabain $3 \cdot 10^{-4}$ M (5 donors)	Ouabain 0 M Donor JW	Ouabain 0 M Donor JF
Cell sodium (meq/kg solids)	0 hr	31.4	33.5	31.9
	3 hrs	68.6	60.1	57.2
Sodium net flux (meq/kg solids/3 hrs)		37.2	26.6	25.3
Average sodium fluxes 0—3 hrs (meq/kg solids/hr)	Influx	20.0	21.5	21.9
	Efflux	7.6	12.6	13.5
Extracellular sodium (meq/l)		136	144	140
Cell potassium (meq/kg solids)	0 hr	249.3	247.1	256.8
	3 hrs	221.5	228.7	241.8
Potassium net flux (meq/kg solids/3 hrs)		—27.8	—18.4	—15.0
Average potassium fluxes 0—3 hrs (meq/kg solids/hr)	Influx	0.98	3.4	5.3
	Efflux	10.23	9.5	10.3
Extracellular potassium (meq/l)	0 hr	5.2	4.7	5.5
	3 hrs	10.2	7.5	8.3
Cell water (g/kg solids)	0 hr	1761	1703	1674
	3 hrs	1830	1778	1770
Water net flux (g/kg solids/3 hrs)		69	75	96

Measurements of unidirectional potassium fluxes (Table VII) clearly showed that the inhibiting effect on potassium transport was progressing with time. K influx decreasing throughout 3 hrs incubation in spite of intracellular Na and extracellular K concentrations which in other media would stimulate ouabain sensitive transport.

Discussion

It has previously been established that the magnitude of passive sodium fluxes through the red cell membrane is dependent on the anion species of the medium (Funder and Wieth 1967 b). The present experiments showed that also the rate of active cation transport was dependent on the anions. Active transport of sodium and potassium was stimulated when red cells were incubated in the bicarbonate

TABLE VII Time course of the unidirectional fluxes of potassium of human erythrocytes suspended in the sodium thiocyanate medium in the absence of ouabain (38 C pH 7.40) Note that influx decreased with time Further explanation is found in the text

Donor	Period (hrs)	Extracellular potassium (meq/l)	Intracellular sodium (meq/kg cell water)	Influx	Efflux	Net flux
				(meq/kg cell solids hr)		
J W	0—1	4.7—5.6	19.7—25.4	3.7	9.6	—5.9
	1—2	5.6—6.5	25.4—29.2	3.5	9.1	—5.6
	2—3	6.5—7.5	29.2—33.8	3.0	9.9	—6.9
J F	0—1	5.5—6.3	19.1—23.6	5.9	10.5	—4.6
	1—2	6.3—7.3	23.6—28.5	5.2	10.7	—5.5
	2—3	7.3—8.3	28.5—32.3	4.8	9.7	—4.9

medium, and the stimulation appeared to be caused by the increasing intracellular sodium concentration Sodium and potassium transport was reversibly inhibited by thiocyanate ions The experiments further showed that the effect of anions on passive cation fluxes (sodium influx and potassium efflux) was independent of the presence of ouabain In the following discussion special attention will be given to the stimulation of active transport caused by bicarbonate and to its possible relation to the increased rate of glycolysis

Sodium and potassium fluxes in the presence of bicarbonate

Accumulation of sodium potassium and water took place when fresh red cells were incubated in a 142 mM $NaHCO_3$ medium (Table I) The changes found were apparently all secondary to the increased influx of sodium and the sequence of events can be described as follows (1) The increasing intracellular sodium concentration stimulated the active extrusion of sodium but the efflux of Na did not reach the level of about 20 meq per kg solids per hour, which would be necessary if the intracellular Na concentration should be stationary (2) The increased influx of sodium was initially accompanied by an increased influx of potassium Potassium transport was apparently stimulated by the intracellular sodium concentration (*cf* Fig 3), demonstrating a tight coupling between sodium efflux and potassium influx as originally suggested by Harris and Maizels (1951) (3) The magnitude of the water net flux was an indirect evidence that the accumulation of sodium and potassium was accompanied by an almost equivalent net flux of anions (Funder and Wieth 1966 a) This was also supported by the fact that the joined concentration—and thereby the osmotic contribution—of (Na + K) was constant during incubation (Table I)

The constancy of the distribution of radioactive chloride between cells and medium makes it likely that the membrane potential of the red cells remained constant during the whole experiment The values of the chloride distribution ratio shown in Table I correspond to membrane potentials of −8 to −11 mV approximately the same magnitude as can be calculated from the distribution of chloride between

red cells and plasma at a pH of 7 40 (Funder and Wieth 1966 a) In another experiment of 8 hrs duration the calculated membrane potential was −13 5 mV (mean value of 6 determinations, S D 1 1 mV)

The results shown in Fig 2 and 3 proved that there is no need to suppose that the properties of the ouabain sensitive transport mechanism are modified when chloride is replaced by bicarbonate The S-shape of the graph in Fig 2 is in accordance with the results obtained in bicarbonate free chloride containing medium by Sachs and Welt (1967) and the quantitative agreement between their and our results appear from the fact that the graph was calculated from the results of Sachs and Welt (1967) A maximal potassium transport of about 6 meq potassium per kg cell solids per hr at slightly elevated intracellular sodium concentrations and an extracellular potassium concentration of 1 4 meq/l required for half maximal stimulation of ouabain sensitive potassium influx are in agreement with the results found in the presence of chloride by Garrahan and Glynn (1967 b) Also the stimulation of active transport by intracellular sodium (Fig 3) may be compared with results found by other investigators in chloride containing media An S shape similar to that seen in Fig 3 was observed by McConaghey and Maizels (1962) for the curve relating sodium efflux to intracellular sodium concentration. Our value of half maximal stimulation of potassium influx (15 meq sodium per kg cell water) agrees with the results of McConaghey and Maizels (1962) and of Whittam and Ager (1965) who employed electrolyte media containing 0 14—0 16 M chloride The value of half maximal stimulation of active cation transport found by Post and Jolly (1957) and by Post *et al* 1960 in media containing 0 15 M chloride was 20 meq/l cells (corresponding to about 30 meq/l cell water) However the cells employed in their studies had been stored for 4—63 days at 2° C and it was reported that the transport capacity was decreased by storage

By means of the findings shown in Fig 2 and 3 it is now possible to explain the time course of the potassium influx (Table I Fig 2 shows that a decrease of extracellular potassium concentration will only cause a minor reduction of potassium influx, as long as the potassium concentration is above 2 meq/l The stimulation of potassium influx caused by intracellular sodium is increasing steeply when the intracellular sodium concentration is raised from 15 to 30 meq kg cell water (Fig 3 The stimulation of potassium transport caused by intracellular sodium was therefore sufficient to maintain a net flux into the cells during the first 6 hrs of the experiment shown in Table I in spite of the decreasing extracellular potassium concentration. During the last 3 hrs of the experiment the potassium concentration had decreased to a level at which a further decrease of extracellular potassium concentration would be followed by reduction of potassium influx Fig 2 The increasing sodium concentration could not maintain a high potassium influx in this period This is understandable because an increase of intracellular sodium concentration above 30 meq/kg cell water has only a slight effect on potassium influx Fig 3) Therefore the potassium fluxes finally settled in a steady state in which no net flux of potassium took place

The existence of a slowly exchangeable fraction of intracellular sodium has been suggested repeatedly (Sheppard, Martin and Beyl 1951, Gold and Solomon 1955) As discussed elsewhere (Funder and Wieth 1966 b) we believe that there has been a tendency of flame photometric methods to overestimate the sodium contents of red cells,—leading to an underestimation of the specific activity of sodium in labelled cells As illustrated by Fig 1 complete exchange of intracellular sodium was achieved in the bicarbonate medium in the experiments which were continued for 6 hrs or more. The total exchangeability of intracellular sodium is not a singular feature of red cells suspended in a bicarbonate medium Garrahan and Glynn (1967 a) have recently shown that the specific activity of sodium lost from preloaded red cells into a chloride Ringer's solution was 98 per cent of the specific activity of total cell sodium

The effect of ouabain on Na and K fluxes, and on glycolysis

The results of Table II, III, VI, and VII show that neither sodium influx nor potassium efflux were influenced by ouabain, when red cells were incubated in bicarbonate or in thiocyanate medium The reduction of potassium influx and of sodium efflux caused by cardiac glycosides has been employed by many investigators as a measure of active cation transport, ever since it was observed that the potassium influx of human red cells was inhibited by low concentrations of ouabain (Schatzman 1953) Determination of 'active' fluxes in this way is only permissible if it can be established that the passive fluxes of sodium and potassium are not affected by the glycoside This cannot be taken for granted The 'downhill fluxes of sodium and potassium appear to be affected by ouabain under certain conditions Glynn (1957) and Garrahan and Glynn (1967 b) thus found a 25 per cent decrease of potassium efflux from red cells suspended in an unbuffered NaCl medium in the presence of cardiac glycosides It has also been reported that sodium influx was decreased by ouabain when red cells were incubated at an extracellular potassium concentration below 5 meq/l (Garrahan and Glynn 1967 c)

The fact that ouabain did not affect the magnitude of the passive cation fluxes was therefore an essential prerequisite for the determination of the quantitative relation between the rates of glycolysis and of active cation transport Because the glucose consumed appeared almost quantitatively as lactate (Table V) it was assumed that the concentrations of glycolytic intermediates were in a steady state so that the relation between ion transport and metabolism could be calculated from the results of Table III and V The rate of active sodium extrusion during the first 3 hrs of incubation in the bicarbonate medium (8 6 meq/kg solids hr) was 70 per cent higher than the rate of sodium transport of red cells incubated in plasma (Funder and Wieth 1967 a) *However the energy utilization of the transport mechanism was in* both cases close to 1 mole of ATP split per 3 moles of sodium transported This observation contributes to the sum of evidence showing that the energy requirement of the sodium transport is independent of the rate of transport and of the electrochemical gradient of the ion transported (*cf* Whittam and Ager 1965)

The effect of thiocyanate on active transport of Na and K

It was hoped that it would be possible to characterize the active transport of Na and K closer by studying the transport in cells whose membrane permeabilities to passive potassium and sodium fluxes were both increased in the presence of thiocyanate However, the rate of active transport was progressively depressed during the incubation (Table VII) Both the action of SCN on permeability and on active transport are fully reversible We have observed that the membrane properties are restored if the red cells are washed with a chloride or a bicarbonate containing medium (*cf* Fig 3) The point of interaction with the transport mechanism is not known, although it has been shown that SCN interferes with glycolysis (Funder and Wieth 1967 b), and thereby probably with the production of energy for ion transport Many enzymes are strongly inhibited by SCN and other anions from the hydrating end of the lyotropic series (*cf* Warren, Stowring and Morales 1965) An interference with the hydrolysis of ATP is possible in view of the finding of Durbin and Kaskebar (1965) that the ATP ase activity of gastric mucosa is totally, but reversibly inhibited by thiocyanate concentrations above 100 mM

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A Sound-Attenuating, Electrically Shielded Chamber for Experiments in Sensory Physiology

By

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Abstract

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The construction of a sound-attenuating, electrically shielded chamber for experiments in sensory physiology is reported in short. The values of sound attenuation are given. Electrical shielding and connection to the laboratory computer are explained.

In the study of sensory systems experiments combining both psychophysical and electrophysiological methods have become more frequent in the past years. Such experiments usually demand a chamber which provides adequate sound attenuation as well as electrical shielding. Other chambers of this kind have been reported earlier (Thorpe and Hinde 1956), (Rodda, Watson and Wilson 1963), (Clarke 1965), (Lee 1967), (Hougardy and Desmedt 1967).

A modification of the chambers mentioned above was constructed at a reasonable cost at the Department of Physiology, University of Helsinki. It consists of a double chamber with the inner chamber "floating" on an isolating layer of insulwool in the outer chamber. The inner and outer walls have an air gap of 5 cm in between. The outer diameters of the chambers are 400 cm × 350 cm × 280 cm, the inner diameters 300 cm × 250 cm × 210 cm. The inner wall is covered by layers of insulwool for minimizing echoes. The wall consists of the following layers from out to in: outer wall: chip-board (12 mm), gypsum wall-board (20 mm), insulwool (75 mm), air-space (50 mm); inner wall: chip-board (12 mm), gypsum wall-board (10 mm), insulwool (5 mm), gypsum wall board (10 mm), ironmesh, air-space (20 mm), peg-board (3 mm), layer of insulwool (100 mm), sound absorbing lamels of insulwool (100 mm). The inner wooden door (50 mm), which is covered with insulwool as well as the outer steel door (150 mm) have double windows. The construction of the chamber is given in Fig. 1.

Ventilation of the chamber is achieved with the aid of a roof ventilator. The metal ventilation tubes for aspirating air in and out are both 2.5 m in length and have an insulwool lining inside to absorb sound (inner diameter 250 mm). The efficiency of this ventilation system has proved to be excellent and causes practically no disturbance.

TABLE I Acoustical properties of the chamber

Frequency (Hz)	Attenuation (dB)	Background noise SPL (dB)	
		Ventilation on	Ventilation off
31.5	19	55	53
63	29	48	47
125	55	28	25
250	72	16	11
500	71	13	9
1000	82	10	9
2000	76	11	11
4000	74	13	13
8000	70	14	14

Sound attenuation of the wall was measured by a precision sound level meter (Bruel & Kjaer, Type 2203 with an Octave Filter Set Type 1613) with a noise source of some 80 dB (Bruel & Kjaer Random Noise Generator, Type 1402). Sound attenuation for the different octave bands is given in Table I. The background noise inside the chamber measured under normal conditions amounted to an average of 10 dB at speech frequencies (500—2000 Hz). A slight increase in the background noise of some 1—2 dB took place when the ventilation was switched on (see Table I for the values for different frequencies). The high values of background noise and the low values of attenuation at low frequencies play no significant role since the sensitivity of the ear at these frequencies is of the order of tens of dBs less than at speech frequencies.

The echotime of bursts of white noise inside the chamber was recorded on a tape recorder (Nagra III) and analysed by a Brüel & Kjaer frequency analyser (Type 2107). In average the echotime amounted to some 0.2 sec, a value barely measurable with the instruments used. This provides possibilities for free field acoustical experiments.

The electrical shield consists of an iron mesh (36 gauge galvanized wire, 12 wires/inch) which covers walls and door of the inner chamber (Fig. 1). 26 Coaxial cables with BNC receptacles mounted on perspex panels, a shielded power line with an inside wall-outlet and 6 iron tubes (1 and 1 1/2 Ø) which are interrupted between the walls and closed by rubber plugs at both ends provide the different connections between inside and outside. The common earth point of the shielding system is behind the outer panel, which additionally holds a laboratory terminal of the institute's computer (μ LINC). In order to avoid earth loops each shield of the 26 coaxial cables can be disconnected from the common earth point by switches on the outer panel. This shielding system effects an 80 dB reduction of the ambient 50 Hz electric stray field and the attenuation of a 50 kHz magnetic field is better than 30 dB.

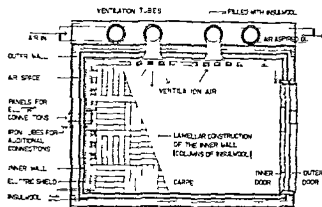


Fig 1 Longitudinal section of the chamber

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Ion Movements in Red Cells Treated with Propranolol

By

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Abstract

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Addition of propranolol to a suspension of human red cells was found to cause outflow of K^+ ions and, to a lesser degree, inflow of Na^+ ions, until a new stationary state was reached in which their cell/medium concentration ratios did not deviate much from unity The cell volume decreased [illegible]

A variety of chemically unrelated substances cause the human red cell membrane to leak alkali ions (Passow 1964) Since the cells usually begin to lyse at higher concentrations, the effect is called prolytic (Davson and Danielli 1938, Ponder 1948) For example, primaquine induces a leakage of potassium, associated with an almost equivalent entry of sodium, and a simultaneous gain of water (Weed, Eber, and Rothstein 1961) Certain other agents, *e g* , lead salts (Joyce, Moore, and Weatherall 1954, Grigarzik and Passow 1958) and valinomycin (Tosteson *et al* 1967), cause a loss of potassium and shrinkage of the cells, while the entry of sodium is low The present paper shows that similar results can be brought about by a beta adrenergic blocking drug propranolol

Methods

[illegible]

suspension was incubated in a water bath at 38 °C, mixed gently and equilibrated with a humidified gas containing 95 % O_2 and 5 % CO_2.

The drugs tested were propranolol (isopropyl-aminonaphthyloxy propanol), pronethalol (naphthyl isopropylaminoethanol), and inpea (nitrophenyl-isopropyl aminoethanol) as their [illegible]

propranolol, when the bulk cations had practically attained the new propranolol induced stationary state. The movements of sodium tracers in the non-stationary state were also studied with the double isotope technique. The red cells were incubated with ^{22}Na for 2.5 hrs. Thereafter they were washed and resuspended in the buffer containing ^{24}Na and propranolol.

From time to time, an aliquot of 2.0 ml of the cell suspension was pipetted into an isotope counting tube and centrifuged at 2000 g for 5 min. A 0.5 ml sample of the supernatant was pipetted into another counting tube, and the remaining supernatant carefully removed without disturbing the cell column. The cells were then washed once with 15 × volume of cold buffer. For determination of the cell volume in the test sample the relative packed red cell volume of the suspension was measured separately, using microcapillary hematocrit centrifugation.

The concentrations of radioactive tracers were determined with an automatic well type crystal scintillation spectrometer (Nuclear Chicago, model 8742). Usually one short acting and one long acting isotope were present, and these were separated with the aid of their different half lives and radiation energies. The ^{22}Na, ^{86}Rb, and ^{137}Cs were supplied by the Radiochemical Centre, Amersham, and ^{24}Na, ^{42}K and ^{82}Br by the Reactor Laboratory, Technical University, Otaniemi. The specific activities of ^{86}Rb, ^{137}Cs and ^{82}Br were high, so that the system contained only very low concentrations of the corresponding non radioactive ions.

Lithium has no suitable radioactive isotope, but it is possible to determine low concentrations of non radioactive lithium with an atomic absorption spectrophotometer (Perkin Elmer, model 303 was used). The addition of 2.5 mmoles of lithium chloride per litre of suspension was found to be sufficient for the determination of lithium both in the cells and in the medium. In some experiments ^{22}Na and ^{42}K were added simultaneously with lithium chloride. It was found that the behaviour of the radioactive tracers was similar to that in the experiments without lithium. This suggests that the concentration of lithium was low enough not to change the system, thus permitting the application of tracer equations.

Concentrations of non radioactive sodium and potassium ions were determined with an internal standard flame photometer (Baird Atomic KY 2 or Beckman 105).

Lactate was measured enzymatically with the LDH NAD system from the whole cell suspension, after precipitating the proteins with trichloroacetic acid. Reagent kits of Boehringer & Soehne Mannheim were used.

Hemoglobin was determined with the cyanmethemoglobin method.

Red cell water was determined by drying to constant weight.

Tracer fluxes in a two-compartment system

General case. The tracer rate constants k_{in} and k_{out} for inward and outward movements respectively are independent of the low tracer concentrations but do depend on the concentrations of all the bulk substances. The constants can be obtained at any time t by simultaneously determining the tracer flows in opposite directions using two tracers of the same ion species, or by determining the flow of one tracer and the simultaneous opposite flow of the corresponding bulk ion. The ratio k_{in}/k_{out} is the cell medium concentration ratio which the tracer tends to attain at time t. It can be calculated as follows

$$\frac{k_{in}}{k_{out}} = \frac{A - r}{1 - Ar} \quad (1)$$

in which r is the cell medium concentration ratio of the inwardly flowing tracer at time t, r' is the medium cell concentration ratio of the tracer or bulk ion flowing at the same time in the opposite direction, and A is

$$A = \frac{V_e}{V_i} \cdot \frac{\frac{1}{1 - R}\frac{dR}{dt}}{\frac{1}{1 - R'}\frac{dR'}{dt}} \quad (2)$$

in which V_e and V_i are the volumes of the medium and the cell at time t, R is the cell medium ratio of total amount of inwardly flowing tracer and R' is the medium cell ratio of total amount of

. .. — . . . the same time in the opposite direction Tangents dR/dt and dR'/dt
' against time
nt stationary state during the change of the
er inflow experiments and that of [24]Na in the
tracer outflow experiments were zero some minutes after addition of propranolol We find that at *this time the term A is zero, and $k_{in}/k_{out} = r$ for [42]K and [86]Rb, and $k_{in}/k_{out} = r'$ for [24]Na*

The tracer rate constant k_{out} at time t is

$$k_{out} = \frac{V_o}{V_i}\,\frac{1}{1+R}\,\frac{\frac{dR}{dt}}{\frac{k_{in}}{k_{out}} - r} \tag{3}$$

The k_{in} can be calculated from the values of k_{out} and k_{in}/k_{out}

The flow J of the corresponding bulk ion is

$$J = k_{in}\,c_o - k_{out}\,c_i \tag{4}$$

in which c_o and c_i are the concentrations of the bulk ion in the medium and in the cell at time t

Stationary state When the distribution of bulk substances is stationary, the tracer rate constants are also time independent, and can be obtained by determining the tracer flow in one direction only A plot against time of the term B,

$$B = \ln \frac{1 + r\,V_i/V_o}{1 - r/r^{\infty}} \tag{5}$$

in which r^{∞} is the cell/medium concentration ratio of the tracer at infinite time and r, V_i, and V_o have the same meaning as before, then gives a straight line From its slope the tracer rate constants k_{in} and k_{out} can be calculated

$$k_{in} = k_{out}\,r^{\infty} = \frac{B\,r}{1/r + V_i/V_o} \tag{6}$$

Results

Flows of non-radioactive sodium and potassium ions and water Initially, the mean cell and medium sodium concentrations were 7 mM and 144 mM and those of potassium 93 mM and 4 7 mM, respectively, *corresponding to a cell/medium concentration* ratio of 0 05 for sodium and 20 for potassium Fig 1 shows that when propranolol was added to the medium, the cell/medium concentration ratio of sodium increased, whilst that of potassium at first decreased rapidly and then slowly approached a new time-independent state With $5\,5 \times 10^{-4}$ M *propranolol the stationary state* cell/medium concentration ratios were 0 25 for sodium and 0 8 for potassium Taking into account that the water concentration of the propranolol-treated cells was 590 g/litre cells, we find that this ratio, when calculated from concentrations per volume of water, was 0 4 for sodium and 1 3 for potassium

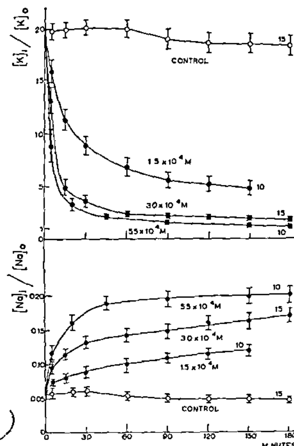


Fig 1 Cell (i)/medium(o) concentration ratios of non radioactive sodium and potassium ions after addition of varying concentrations of propranolol Numbers of experiments and means ± S D are given

The cell volume was decreased 20 % by 5 5 × 10⁻⁴ M propranolol (Fig 2)

The ion flows calculated from the concentration and volume changes are shown in Fig 3 During the first few minutes at 5 5 × 10⁻⁴ M propranolol the outflow of potassium was about 120 (mmole/litre initial cells)/hr, and the inflow of sodium about 30 (mmole/litre initial cells)/hr At the end of the experiments the loss of potassium salts exceeded the gain of sodium salts by 50 mmoles, 30 mmoles, and 13 mmoles per litre of initial cells with three decreasing concentrations of propranolol

In the control experiments only small deviations from the initial ion concentrations and cell volumes occurred

Tracer movements during the non-stationary state

Fig 4 shows the tracer distribution ratios as a function of time when the tracers and propranolol were added to the medium simultaneously

The entries of both Li and ^{22}Na were accelerated and at the end of the experiments the cell/medium concentration ratios were higher in the treated cells than in the controls The accumulation of ^{137}Cs was also accelerated, but at the end of the

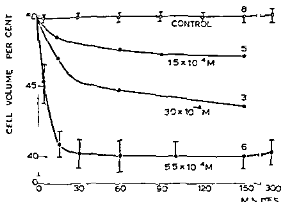


Fig 2 Packed red cell volumes as per cent of volumes of the whole suspension after addition of propranolol

experiments the cell/medium ratio, when compared with that of the controls, was lower for ^{137}Cs than for Li or ^{22}Na

The entries of ^{42}K and ^{86}Rb were very rapid, so that even in the first sample at a propranolol concentration of 5 5 × 10⁻⁴ M the ^{42}K concentration in the cell was 3 times that of the medium Thereafter the tracer began to leak out The peaks of ^{42}K were 1 6 times as high as those of ^{86}Rb Their heights depended on the concentration of propranolol In experiments which were carried out as rapidly as possible, it was found that the peaks were reached in about 3 min for ^{42}K and 6 minutes for ^{86}Rb The accumulation rates of ^{42}K and ^{86}Rb were sensitive to the initial nonradioactive potassium ion concentration of the medium With a potassium concentration of 20 mM the cell/medium concentration ratio of ^{42}K was only about 0 7 in the first sample with 5 5 × 10⁻⁴ M propranolol and no peak accumulation occurred

The effect of propranolol on the outflow of ^{22}Na is depicted in Fig 5 The outflow was low during the first few minutes, and so the curve was sigmoid If the initial medium/cell concentration ratio of ^{22}Na was about unity, the ^{22}Na first flowed into the cell and only thereafter began to flow out

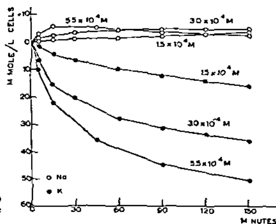


Fig 3 Flows of non radioactive sodium and potassium ions as mmoles per litre of initial cells after addition of propranolol

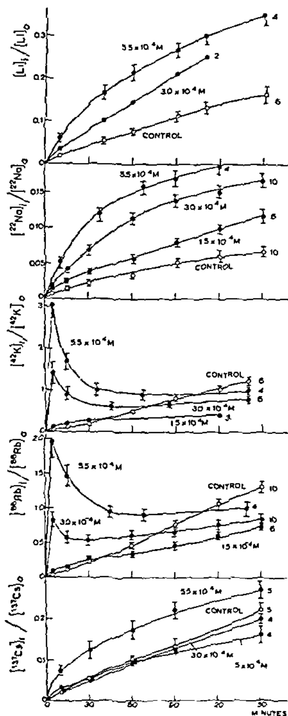


Fig 4 Cell/medium concentration ratios of tracer ions added to the medium simultaneously with propranolol

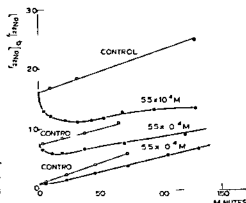


Fig 5 Medium/cell concentration ratios of ^{24}Na in the outflow experiments at various initial medium/cell concentration ratios The rate of entry of ^{24}Na in these experiments was slightly higher than that of ^{22}Na in the experiments described in Fig 4

Fig 6 shows that immediately after addition of propranolol the k_{in}/k_{out} of sodium tracer, that is, the cell/medium concentration ratio which the tracer tends to attain, as calculated from the inflow and outflow experiments according to equations (1) and (2), increased and reached a maximum which was greater than unity The ratio of potassium tracer rapidly decreased but remained slightly higher than that of sodium tracer Just after addition of propranolol the k_{in}/k_{out} values of both cationic tracers were higher than the medium/cell ratio of bromide tracer Since anions permeate the red cell membrane very rapidly, this ratio may be considered directly to represent the k_{out}/k_{in} ratio, and the distribution of passive ions in the flux ratio analysis of Ussing (1949) We see that it was remarkably constant At the end of the experiments it lay between the k_{in}/k_{out} values of cationic tracers, close to that of potassium

The tracer rate constants and ion flows 5 min after addition of propranolol,

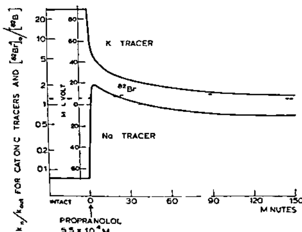


Fig 6 The ratio of inward to outward rate constants of sodium and potassium tracers and the medium/cell concentration ratio of bromide tracer Values are given per volume of water The millivolt scale is calculated according to the Nernst equation

TABLE I Tracer rate constants, and concentrations and flows of non-radioactive ions in the non-stationary state 5 min after the addition of propranolol (5 5 × 10⁻⁴M) The values are given per actual cell volume

	Na^+	K^+
k_{in}, hr⁻¹	0 4	5
k_{out}, hr⁻¹	0 3	2 5
c_i, mmole/litre	15	93
c_o, mmole/litre	130	12
k_{in} c_o, (mmole/litre cells)/hr	50	60
k_{out} c_i, (mmole/litre cells)/hr	4 5	230
Flow, k_{in} c_o—k_{out} c_i, (mmole/litre cells)/hr	45	—170

calculated according to equations (1)—(4), are given in Table I. Corresponding values for intact cells are given in Table II For sodium tracer, the k_{in} was 16 times and k_{out} 0 53 times that of the control For potassium tracer, the k_{in} was 50 times and k_{out} 100 times that of the control The bulk ion flows given in Table I, even when they are changed to results per volume of initial cells, are slightly higher than the flows calculated directly from the concentration and volume changes shown in [illegible] 3 The discrepancy may well arise from inaccuracies in the determination of the [illegible]idly changing non-stationary concentrations

Tracer movements during the stationary state.

Tracer rate constants and their inward to outward ratios, calculated according to equations (5) and (6) from the experiments in which the tracers were added three hours after the addition of propranolol, are given in Table II These rate constants

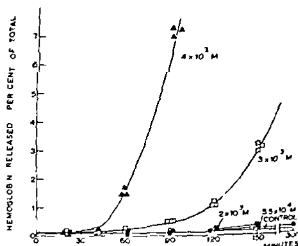


Fig 7 Hemolytic breakdown of the cells at various concentrations of propranolol

TABLE II Tracer rate constants and distributions, and concentrations of non radioactive sodium and potassium ions in the intact and propranolol (5 5 × 10^{-4} M) induced stationary states The values are given per actual cell volume, and are means of 3 to 6 expts The stationary concentrations of non radioactive sodium and potassium ions were calculated from the ion concentrations of the whole cell suspension, the packed red cell volume, and the stationary distribution ratio of the corresponding tracer

	Li^+		Na^+		K^+		Rb^+		Cs^+	
	Control	Propranolol	Control	Propranolol	Control	Propranolol	Control	Propranolol	Control	Propranolol
k_{in} hr^{-1}	0 065	0 12	0 025	0 075	0 25	0 66	0 25	0 56	0 046	0 18
k_{out} hr^{-1}	0 11	0 24	0 57	0 30	0 025	0 86	0 021	0 70	0 015	0 40
k_{in}/k_{out} = $^*c_i^\infty/^*c_o^\infty$	0 56	0 50	0 045	0 25	10	0 77	12	0 80	3 0	0 40
c_i, mmole/litre			6 3	27	89	41				
c_o mmole/litre			145	110	8 9	54				
k_{in} c_o = k_{out} c_i, (mmole/litre cells)/hr			3 6	8 3	2 2	35				

were high as compared with those of the controls, their order being ^{42}K > ^{86}Rb > ^{137}Cs > Li ≥ ^{22}Na However, they were not so high as those found just after addition of propranolol (Table I) The k_{in}/k_{out} ratios did not deviate much from unity in the treated cells

Hemolysis Hemolytic breakdown of the control cells was 0 25 ± S E 0 02 % at the end of the 6 hour incubation period, and was not significantly changed by the propranolol concentrations used to evoke the cation shifts However, marked hemolysis began after a certain lag period, when the propranolol concentration was 3 × 10^{-3} M or higher (Fig 7)

Lactic acid production Lactic acid production was only slightly changed During the first hour after addition of propranolol (3 × 10^{-4} M) the mean lactic acid production ± S E of 7 expts was 4 88±0 073 (mmoles/litre initial cells)/hr, while that of the controls was 4 26 ± 0 048 (mmoles/litre initial cells)/hr

Effect of other similar drugs Pronethalol in 3 expts with 7 5 × 10^{-4} M and in 3 expts with 1 5 × 10^{-3} M had effects similar to one fifth of the concentration of propranolol upon the distributions of non-radioactive sodium and potassium ions, packed red cell volumes, and ^{86}Rb accumulation Inpea in concentrations from 1 × 10^{-4} to 3 × 10^{-3} M did not exert any measurable effect

Discussion

Propranolol and pronethalol were found to cause outflow of potassium salts from cells and, to a lesser degree, inflow of sodium salts, whilst inpea was without this effect These three drugs all possess beta-adrenergic blocking properties, but

and pronethalol differ from inpea in that they abolish cardiac arrhythmias due to digitalis glycosides (Somani and Lum 1965, Turner 1966), are strong local anaesthetics (Morales-Aquilera and Waughan Williams 1965, Murmann, Saccani Guelfi, and Gamba 1966), and have a high chloroform/water partition ratio (Almirante *et al* 1966) Propranolol leads to loss of potassium from the isolated rat diaphragm also (Manninen 1968) Valinomycin, which affects the red cells in a similar way to propranolol, increases the permeation of rubidium and potassium ions even through artificial bimolecular lipid membranes (Lev and Buzhinsky 1967, Mueller and Rudin 1967, Andreoli, Tieffenberg, and Tosteson 1967)

Against the outflow of bulk potassium ions, potassium and rubidium tracer ions were rapidly taken up into the cells, reaching levels greatly exceeding their final concentrations A similar counter-current transport has been demonstrated during non-stationary states in the nerve (Keynes and Lewis 1951) and in the red cells (Joyce *et al* 1954, Grigarzik and Passow 1958) Joyce and his co-workers suggested that the transient accumulation of potassium tracers in red cells losing bulk potassium after exposure to lead chloride might be due to compensatory acceleration of an "inward potassium pump" *In our experiments, a few minutes after addition of* propranolol, the product of the outward rate constant of potassium tracer multiplied by the concentration of bulk ion in the cell was 230 (mmole/litre cells)/hr, and the product of the inward tracer rate constant multiplied by the concentration of bulk ion in the medium was also high, 60 (mmole/litre cells)/hr At that time the bulk concentrations were but little changed, so that for bulk potassium there was still steep concentration gradient from the medium to the cell The inward rate constant of sodium tracer increased to the extent that the ratio of the inward to outward rate constants of this tracer transiently exceeded unity and even the bromide distribution ratio Indeed, according to the popular terminology the results would mean that propranolol increased not only the "leak" but also the "pump" But, in agreement with Sims (1966), we do not consider it meaningful to attempt resolution of ion flow into a fraction due to 'active transport and another simultaneously due to "passive" diffusion

However, the accelerated movements of tracer ions during the non stationary state deserve consideration As stated by Heckmann and Passow (1967), since the red cell *membrane is very permeable to anions a diffusion potential due to the flows of* bulk salts cannot account for the rapid uptake of tracer ions

A "carrier' exchanging cations especially potassium, across the cell membrane, its action greatly accelerated by addition of propranolol would explain the results It is not necessary that the carrier should form a chemical compound with the ion If there is a circulation of water in the membrane, this would act as a carrier It has been suggested that circulation of water might result from its coupling with metabolites (Ingraham, Peters and Visscher 1938 Schlogl 1957 Salminen, Ekman, and Manninen 1968) During the stationary state only nutrients and end products flow in relation to the total membrane but driven by them water and substances carried by it may be flowing in and out through certain regions This circulation, and thus

its exchange effect, would be increased during the non stationary state by the coupling of water with bulk salts flowing down their gradients A coupling of this type between the flows of bulk ions and tracer ions would occur in a model constructed by Nims (1967), if tracers were added Such a system requires that the membrane is not homogeneous, but has different properties in at least two regions The rate constants and their inward to outward ratios for different tracers may differ, depending on the frictional coefficients of these tracers with respect to water and different regions of the membrane

The ratio of inward to outward rate constants of potassium tracer, when divided by the corresponding ratio of sodium tracer, yields a quotient which indicates the cells' ability to accumulate potassium in preference to sodium This is some hundreds in intact cells, and was three in the treated cells Under conditions that do not involve a change in the structure of the cell membrane, it is related to the rate of lactic acid flow (Thurber and Thompson 1967) However, propranolol seems to have added to the sites through which cations can pass, and since this must have increased the effective area of the membrane, the deviation from equilibrium cannot be expected to have been as great as in the intact cell, even though lactic acid production, when calculated per unit volume of cell, was not decreased

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Effects of Increased Intracranial Pressure on Cerebral Blood Flow and on Cerebrospinal Fluid HCO_3^-, pH, Lactate and Pyruvate in Dogs

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Abstract

KJALLQUIST, A, B K SIESJO and N ZWETNOW *Effects of increased intracranial pressure on cerebral blood flow and on cerebrospinal fluid HCO_3^-, pH, lactate and pyruvate* Acta physiol scand 1969 75 345—352

The cerebral perfusion pressure was reduced in dogs by infusing an artificial cerebrospinal [illegible] the mean arterial pressure by means of [illegible] CSF concentrations [illegible] reductions (25—40 [illegible] ued by an increased [illegible] by a decreased bi [illegible] ght hypoxic lactacidosis in spite of [illegible] When the cerebral perfusion pres [illegible] and often very marked lactacidosis [illegible] in the CSF there was a good agreement between the *increase in the lactate, and the decrease in the bicarbonate concentration* but with a marked and longlasting lactacidosis the decrease in the bicarbonate concentration could exceed the lactate accumulated The possible coupling between the lactacidosis and the decrease in the cerebrovascular resistance is discussed

In a preceding paper (Kjallquist, Siesjo and Zwetnow 1969) experiments were reported in which the cerebral perfusion pressure was acutely reduced by means of an induced increase in the cerebrospinal fluid (CSF) pressure It was found that pronounced reductions of the cerebral perfusion pressure were accompanied by marked changes in cerebral venous blood These changes consisted of decreases in pO_2 and pH and of increases in pCO_2 lactate, pyruvate, and in the lactate/pyruvate ratio during the period of increased CSF pressure When the intracranial pressure was brought back to normal a reactive hyperemia usually appeared, and the pO_2, pCO_2 and pH in cerebral venous blood varied in accordance with the hyperemia However irrespective of the severity of the preceding pressure reduction, there were

no persisting lactate and pyruvate changes in the venous blood once a normal pressure had been restored

In order to elucidate the lactate/pyruvate changes observed in cerebral venous blood during an intracranial hypertension the lactate, pyruvate and bicarbonate concentrations in cisternal CSF were measured and related to the concomitant changes in cerebral blood flow Such measurements should be of potentially greater usefulness than measurements on blood since the CSF lactate/pyruvate ratio appears to be a sensitive indicator of tissue hypoxia in the brain (Siesjo, Kjallquist and Zwetnow 1968, see also Granholm and Siesjö 1968), and since the brain extracellular hydrogen ion concentration has been suggested to influence cerebral vascular resistance (see Discussion) *A preliminary report on some of the present findings has* been published (Zwetnow, Kjallquist and Siesjo 1968)

Methods

The present results were obtained on 22 *mongrel dogs which* were either anaesthetized with Nembutal (40 [illegible]
Fluothane (ICI [illegible]
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Celocurin kloric [illegible] and *ventilated artificially* Catheters were introduced into one femoral artery for continuous blood pressure recording and *for removal of arterial samples* into the superior sagittal sinus for sampling of cerebral venous blood and into the left vertebral artery for injections of Xenon[133] dissolved in Ringer's solution

Arterial pO_2 pCO_2 *and pH* were *measured* with *microelectrodes. Cortical blood flow* (CBF grey) was calculated from the tissue clearance of Xenon[133] A double barrelled needle was *introduced percutaneously into the* cisterna magna and used for infusion of artificial CSF from a reservoir *for recording of the CSF pressure* and for removal of CSF samples The [cer]ebral perfusion pressure was calculated as the difference between the mean arterial and the [CS]F pressure *For details of techniques and experimental procedures reference* is made to a [p]receding paper (Kjallquist Siesjo and Zwetnow 1969)

The CSF pressure was increased by infusing an artificial CSF into the cisterna magna Since the artificial CSF infused did not contain any lactate or pyruvate and a fixed bicarbonate concentration *(24 7 mEq/l) the CSF samples collected immediately* after a pressure period had low lactate and pyruvate concentrations and a bicarbonate concentration which was close to that of *the artificial CSF In order to allow a* rough calculation of the lactate pyruvate and bicarbonate concentrations in the native CSF carboxyl C^{14} inulin was *added to the artificial CSF and its admixture to the samples recovered was calculated* from the dilution of the inulin after liquid scintillation counting in a Packard Tri Carb scintillator *However this correction could only* be assumed to give approximate values for the parameters mentioned especially since lactate and pyruvate appear to exchange very rapidly between the *tissue and CSF (see Results) For these* reasons the corrections could only be assumed to give reasonably correct results within the first 30—60 minutes after the pressure had *been restored* to normal In order to clarify this point further four experiments were carried out with re*ductions of the cerebral perfusion* pressure by means of arterial bleeding This was done by withdrawing arterial blood into heparinized 100 ml glass syringes until the desired mean *arterial pressure had been reached A normal perfusion pressure was then obtained* by reinfusing the blood previously withdrawn

The CSF lactate and pyruvate concentrations were measured enzymatically after collecting the samples directly into liquid nitrogen The CSF bicarbonate concentration was usually measured with a modification of the Conway diffusion method Siesjo 1962 In some experiments however the CSF pH was measured with a Radiometer micro glass electrode after collecting the CSF samples anaerobically into glass capillaries In order to avoid errors in the pH measurements due to the low buffer capacity of *the* CSF repeated measurements were [illegible] of the glass electrode The pH values were referred to the [illegible] tional *Bureau of Standards* 6 841 at 37 5 C In these [illegible] centration was calculated from the Henderson Hassel[illegible] factor of 0 0314 mmole/kg/mm Hg (Siesjo 1962) and [illegible] bert and Carman 196[illegible] The CO_2 tension of the CSF [illegible]
samples was *measured* with a *micro* CO_2 electrode

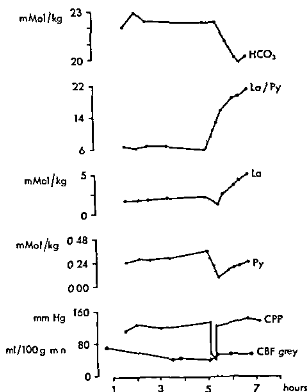


Fig 1 The lactate pyruvate and bicarbonate concentrations in cisternal CSF after an induced increase in the CSF pressure (cerebral perfusion pressure reduced to about 50 mm) related to cortical blood flow (CBF grey) Note constancy in lactate pyruvate and bicarbonate during repeated sampling of CSF and marked changes observed after the pressure period

Results

The present results showed that reductions of the cerebral perfusion pressure by only 25 to 40 mm Hg could lead to significant increases in the CSF lactate and pyruvate concentrations and in the lactate/pyruvate ratios and to decreases in the CSF bicarbonate concentration In all the present experiments the CSF pCO changed very little so that the decrease in the bicarbonate concentration usually implied a corresponding decrease in the CSF pH With more prolonged and severe reductions of the cerebral perfusion pressure which were accompanied by a decreased blood flow and followed by a reactive hyperemia very marked and sometimes very longlasting changes regularly occurred in the lactate and pyruvate concentrations in the lactate/pyruvate ratios and in the bicarbonate concentrations In longlasting experiments it could be found that the lactate/pyruvate ratio remained at very high levels and the bicarbonate concentration at very low levels for periods of several hours in spite of the fact that the cerebral blood flow returned to normal values after the initial reactive hyperemia A reduction of the cerebral perfusion pressure due to a bleeding of the animals (stagnant hypoxia) gave results which were very similar to those obtained after an induced increase in the CSF pressure

Under normal control conditions the lactate and pyruvate concentrations and the lactate/pyruvate ratios of cisternal CSF usually show a small variability both within the same animal and between the individual animals Thus in a series of

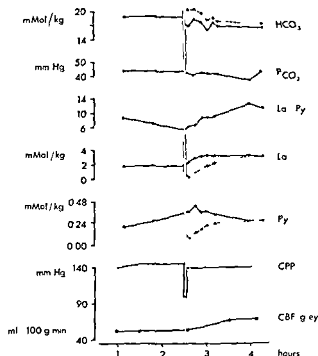


Fig 2 The effects of a moderate increase in CSF pressure (cerebral perfusion pressure decreased from 145 to 100 mm Hg) on the CSF lactate, pyruvate and bicarbonate concentrations The decrease in perfusion pressure was not followed by any immediate change in cortical blood flow (CBF grey) but led to a significant increase in the CSF lactate/pyruvate ratio When corrections were made for the admixture of artificial CSF to the samples analyzed (unbroken lines after the pressure period) the CSF lactate was found to have increased and the bicarbonate to have decreased (see Methods)

19 dogs including the present ones, the mean CSF lactate concentration was 1 79 ± 05 mmol/l the mean pyruvate concentration 0 233 ± 0 009 mmol/l, and the mean lactate/pyruvate ratio 7 8 ± 0 2 This relative constancy is illustrated in Fig 1 which also shows the typical CSF findings in an experiment in which the cerebral perfusion pressure was reduced for 15 min to 50—60 mm Hg It is seen that the lactate, pyruvate and bicarbonate concentrations, as well as the lactate/pyruvate ratio remained stable for several hours until the perfusion pressure was decreased Immediately after the reduction of the perfusion pressure there was a progressive increase in the lactate concentration, and in the lactate/pyruvate ratio and a marked decrease in the bicarbonate concentration In this experiment no correction was made for the dilution of the native CSF with artificial CSF, which means that the *changes in the lactate, pyruvate and bicarbonate concentrations were underestimated* However the dilution should not affect the true lactate/pyruvate ratio It can also be seen that the calculated CBF increased by about 20 per cent after the increase in the CSF pressure, and then remained increased during the rest of the experiment

Fig 2 illustrates an experiment in which the cerebral perfusion pressure was reduced from 145 to about 100 mm Hg for only 3 min This shortlasting and moderate reduction of the cerebral perfusion pressure led to an increase in the CSF lactate concentration and in the CSF lactate/pyruvate ratio and to a decrease in the bicarbonate concentration In this experiment the lactate pyruvate and bicarbonate concentrations in the native CSF were calculated from the dilution of the added inulin The figure also illustrates the increase in CBF which often accompanied

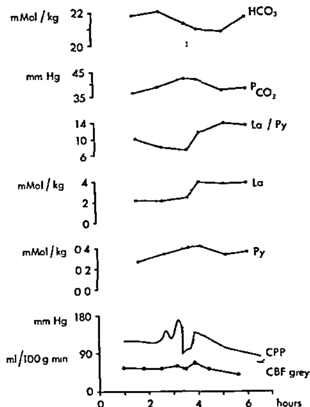


Fig 3 pCO_2 bicarbonate lactate and pyruvate in cisternal CSF after a reduction of the cerebral perfusion pressure (CPP) to about 90 mm Hg induced by arterial bleeding Note increase in the lactate/pyruvate ratio and in the lactate concentration and the corresponding decrease in the bicarbonate concentration

the lactacidosis in the CSF The difficulty of assessing the relevant lactate and bicarbonate concentrations in the mixed CSF withdrawn from the cisterna magna after the period of increased intracisternal pressure was avoided in those experiments in which the cerebral perfusion pressure was decreased by lowering the blood pressure Fig 3 shows an experiment in which the mean arterial blood pressure was reduced from about 140 to 90—100 mm Hg for a period of about 20 min Even in this case there was an increase in the CSF lactate concentration and in the CSF lactate/pyruvate ratio together with a decrease in the bicarbonate concentration

When the cerebral perfusion pressure was moderately reduced or if it was markedly reduced for only a few minutes the chemical changes in the CSF usually were at least partially reversible However with longlasting or repeated reductions of the perfusion pressure there were longlasting and sometimes even progressive lactate/pyruvate and bicarbonate changes Fig 4 illustrates such a finding after two short lasting but pronounced reductions of the perfusion pressure In this experiment the CSF lactate concentration was about 8 mEq/l the lactate/pyruvate ratio about 25 and the bicarbonate concentration about 12 mEq/l five hours after the second pressure period It was seen in several experiments that such a remaining lactacidosis in the CSF could persist when there was a normal or nearly normal cerebral blood flow Thus in the experiment exemplified in Fig 4, the CSF pH remained at values

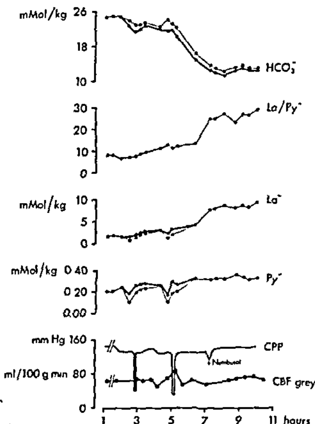


Fig 4 Lactate, pyruvate and bicarbonate in cisternal CSF after two consecutive increases in the CSF pressure (cerebral perfusion pressure reduced to 40 and 30 mm Hg, respectively), related to cerebral blood flow (CBF grey) The lactate, pyruvate and bicarbonate concentrations in the *native CSF were approximately* calculated from the dilution of C^{14} Inulin added to the artificial CSF (unbroken lines, see Methods) Note progressive increase in the CSF lactate/pyruvate ratio, and marked and long lasting changes in the lactate and bicarbonate concentrations in spite of the normalization of CBF

around 7 1 at a time when the CBF had returned to the prehypertensive values

In all experiments with a moderate lactacidosis in the CSF there was a rough correspondence between the increase in the CSF lactate concentration and the decrease in the CSF bicarbonate concentration However, with a marked or longlasting CSF lactacidosis there could be a larger decrease in bicarbonate than the corresponding increase in lactate, indicating that under those conditions the CSF was possibly acidified by some additional mechanism (*Fig* 5)

Discussion

The present results have shown that even moderate increase in the intracranial pressure, leading to a reduction of the cerebral perfusion pressure to values still within the pressure range where autoregulation is ordinarily seen, led to increases in the CSF lactate concentration and in the CSF lactate/pyruvate ratio as well as to decreases in the CSF bicarbonate concentration The results have also shown that when the cerebral perfusion pressure was markedly, or repeatedly, lowered there was a pronounced and longlasting lactacidosis in the CSF with a marked increase in the lactate/pyruvate ratio It is obvious that these metabolic changes were caused by the reduced perfusion pressure *per se* and not by the infusion of an artificial CSF into

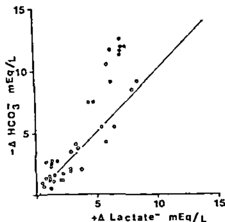


Fig 5 Relation between decrease in CSF bicarbonate and increase in CSF lactate after reductions of the cerebral perfusion pressure in 10 animals (filled circles represent values measured in one single experiment pH 7 08) In order to minimize errors caused by the dilution of the native CSF by artificial CSF (see Methods) only uncorrected values were used from samples drawn 30 min or more after a pressure period A comparison with the line of equality shows that during a marked lactacidosis in the CSF the decrease in bicarbonate exceeded the increase in lactate

the ventriculocisternal system, since the same changes were seen after a reduction of the perfusion pressure due to arterial hypotension and since the ventriculocisternal system can be perfused for hours with artificial CSF without any significant changes in the lactate/pyruvate ratio (Siesjo Kjallquist and Zwetnow 1968)

The increased lactate/pyruvate ratio and the increased lactate concentration in the CSF after a period of decreased cerebral perfusion pressure strongly suggest that the tissue metabolism is or has been changed in the direction of anaerobiosis An unexpected finding was the lactacidosis in the CSF and the increased lactate/pyruvate ratio after such moderate reductions of the cerebral perfusion pressure which usually did not cause any measurable change in CBF These results suggest that a moderate hypoxic lactacidosis may accompany even small reductions of the cerebral perfusion pressure possibly due to a transient lowering of the CBF

The main object of the present work was to study the magnitude and the duration of the CSF lactate pyruvate and bicarbonate changes accompanying decreases in the cerebral perfusion pressure but it is tempting to speculate over a possible coupling between the chemical changes and the changes in the vascular resistance Thus many recent results suggest that the extracellular hydrogen ion concentration in the brain is one of the main factors regulating cerebrovascular resistance Thus it has been reported that the normalization of the CBF in sustained hypocapnia and hypercapnia is acompanied by a tendency for the CSF pH to normalize (Severinghaus 1965 Agnoli 1968) that there is a parallelism between CBF and the CSF hydrogen ion concentration in clinical cases (Skinhoj 1966) and between cortical flow and surface pH of the brain in experimental hypoxia (Betz 1967 Betz and Kozak 1967) and finally that the CBF appears to vary with the CSF H during ventriculocisternal perfusions with solutions of varying pH (Siesjo Kjallquist and Zwetnow 1968) Recently Fencl Vale and Broch (1968) have elegantly shown that CBF varies with CSF pH and not with pCO in chronic non respiratory acidosis and alkalosis in man

Although the results quoted make it logical to assume that the increased CSF

acidity in the present experiments may be partly responsible for the decreased cerebrovascular resistance during and after the decreases in the cerebral perfusion pressure, the present results do not allow any rigid interpretation Thus although the CSF always increased in acidity when a reactive hyperemia was elicited the lactacidosis in the CSF usually outlasted the reactive hyperemia sometimes by several hours The fact that there was no rigid temporal relationship between the changes *in the CBF and in the CSF pH does not exclude, however, that the CSF* pH is an important regulator of the CBF under normal conditions Thus the bulk CSF pH probably does not reflect the true extracellular pH in the non steady state conditions prevailing during and after a marked decrease in the cerebral perfusion pressure Moreover, the regulation of the CBF under pathophysiological conditions (*c f* Petersen and Zwetnow 1967 Freeman and Ingvar 1967) may be different from the normal regulation.

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Acta physiol. scand. 1969. 75. 353—359

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The Influence of Arterial P_{O_2} on Renal Tissue P_{O_2}

By

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Abstract

APERIA, A C *The influence of arterial* P_{O_2} *on renal tissue* P_{O_2} Acta physiol scand. 1969 *75* 353—359

In certain aspects the kidney appears to differ from other organs in the use of oxygen. The relatively low P_{O_2} both of the renal medulla and of the urine has been interpreted to indicate that the medulla receives much less oxygen than the cortex This has generally been considered an effect of the countercurrent system (Lee *et al* 1962) Other studies (Gyorgy *et al* 1928, Dickens and Weil-Malherbe 1936, Kean *et al* 1962, Lee *et al* 1962, Lee *et al* 1962, Herms and Malvin 1963, Winters 1964) have shown that the renal medulla in contrast with most other tissues derives most of its energy from anaerobic metabolism Furthermore, it has been demonstrated in this laboratory (Aperia and Liebow 1964) that with a rise in arterial P_{O_2}, urine P_{O_2} in the dog responds with an initial rise and then a fall, which levels off at a value often below that obtaining during the previously lower arterial P_{O_2} This finding was interpreted to indicate that increasing arterial P_{O_2} results in reduction of blood supply to the renal medulla Such a homeostatic mechanism tending to keep renal medullary tissue P_{O_2} at a constant low level might be of significance for the maintenance of anaerobic metabolism Since this hypothesis is built on the assumption that the urine P_{O_2} is an index of renal medullary tissue P_{O_2} it would be desirable to establish the relationship between arterial and renal tissue P_{O_2} by direct measurement

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Material and methods

The experiments were carried out in mongrel dogs of either sex, weighing 15—20 kg under i v sodium pentobarbital (Nembutal) anesthesia initially at 30 mg/kg, with small supplements as required

The P_{O_2} determinations were made
The electrodes were calibrated at 37°
by contrast bubbling of appropriate g
each experiment The instrument, eq
by the oxygen coefficient of the medium, and the absolute values obtained for tissue P_{O_2} can therefore be considered valid, despite calibration of the electrode in distilled water

[illegible]

between air and 100 % oxygen Steady state values were obtained when the animal had been breathing a constant gas mixture for at least 10 min Generally measurements were made in two different parts of the kidney simultaneously One to five steady state values were recorded for each electrode in the same animal

After an interval varying from 30 minutes to several hours the sensitivity of the electrode tended to decrease, as manifested by a slower response to changes in arterial P_{O_2} or a continuous downslope of the recorded P_{O_2} This was explained on the basis of tissue changes secondary to the implantation of the elctrode At the first sign of such reduced sensitivity the observations were discontinued

Blood samples were withdrawn from a catheter in the femoral artery into sealed syringes for determination of P_{O_2} by a method described previously (Aperia and Liebow 1964)

After sacrificing the animal, the position of the tip of the electrode which was kept *in situ*, was determined accurately first by sectioning the kidney close to the plane of the needle track and after gross inspection and recording of position of the tip of the electrode, embedding the block for histological examination, with care to display the entire length of the track Histological examination also provided essential evidence that the kidney was free of disease

Results

Simultaneous recordings of cortical and medullary tissue P_{O_2} were obtained in 12 normal animals breathing first room air and then 100 % oxygen

In terms of absolute values for tissue P_{O_2} and response of tissue P_{O_2} to changes in arterial P_{O_2}, three different zones of the kidney could be identified cortex, outer medulla, and inner medulla Anatomically, the outer medulla consists mainly of segments of proximal convoluted tubules, thick ascending limbs of the loops of Henle and closely grouped bundles of vessels, while the inner medulla is mainly comprised of thin limbs of Henle s loops and more sparsely arrayed vessels

The mean absolute values for arterial and renal tissue P_{O_2} obtained when the animals had been breathing room air or 100 % oxygen for approximately 10 min are recorded in Table I When the animals were breathing air there was no significant difference between cortical and outer medullary tissue P_{O_2} In 4 of 15 simultaneous observations the P_{O_2} of the outer medulla was in fact found to be slightly higher than the P_{O_2} of the cortex With the rise in arterial P_{O_2} following respiration of 100 % oxygen, there was a highly significant increase in cortical tissue P_{O_2}, while the average P_{O_2} of the outer medulla was actually slightly lower than during air breathing The already extremely low P_{O_2} of the inner medulla was but little affected by the increase in arterial P_{O_2} One of the 13 animals was found to have

TABLE I Relation of Arterial P_{O_2} to Renal Tissue P_{O_2}

	P_{O_2}FA		P_{O_2} cortex		P_{O_2} outer medulla		P_{O_2} inner medulla	
Experiment	Air	O_2	Air	O_2	Air	O_2	Air	O_2
1	95	580	43	90	45	25	—	—
2	92	490	57	112	46	37	—	—
			84		49 5			
3	89	500	40	68	—	—	13	3 5
			48					
4	86	616	18	62	48	32	2	2
			28					
5	93	525	56	340	39	12	—	—
			58		23			
6	88	430	35	150	36	36	—	—
			40	115	42			
					42			
7	92	430	76	94	—	26	4	2
			78	93			3	
8	83	500	68	94	—	—	2	2
			72				1 5	1 5
9	92	475	28	152	—	—	—	—
			30	128				
			29					
10	87	440	40	78	—	—	—	—
			48					
11	89	510	36	210	32*	22	—	—
			47	140	34*	20		
			51		21**	23		
			58		23**			
					24**			
12	82	435	36	145	38	32		
			33	154	38	40		
			30		39			
m±S E M	89±1 2	494±17	47±3 4	131±16	36 4±2 2	27 7±2 5	4 3±1 8	2 2±0 6

All values for P_{O_2} in mm Hg
* ** Values recorded from different electrode positions

an extremely low cortical P_{O_2} of approximately 2 mm Hg during respiration of room air and was without response to 100 % oxygen The kidney proved to be anuric and the low cortical tissue P_{O_2} was interpreted to be the result of renal ischemia, propably caused by traction on the renal pedicle after exposure of the kidney The data from this animal are not included in Table I Continuous recordings of the tissue P_{O_2} during shifts from air to 100 % oxygen breathing and vice versa were made in all twelve animals Typical tracings from various portions

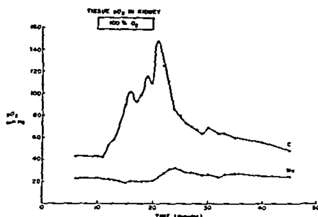


Fig 1 Continuous recordings of tissue P_{O_2} during room air, and 100 % O_2 breathing The duration of the latter is indicated by the length of the horizontal bar Electrode positions — C in the mid-cortex, Mo in the outer medulla just below the corticomedullary junction

of the kidney in one dog are illustrated in Fig 1 and 2 Following respiration of 100 % oxygen, there was a sharp and almost instantaneous rise in cortical P_{O_2} which often continued during the entire 10 minute period on oxygen After the animal was returned to air the cortical P_{O_2} often continued to rise for 1—2 min but then declined with a downward slope almost as steep as the upward In contrast the tissue P_{O_2} of the outer and inner medulla changed only slightly

Discussion

Several problems exist in the determination of tissue P_{O_2}, which indicate caution in interpretation Firstly, while there is no way of precisely standardardizing the electrode *in vivo*, the instrument has been improved, so that it exhibits minimal sensitivity to such factors as diffusion coefficient of the medium and agitation Secondly, it must also be considered whether the measured values represent the P_{O_2} of the tissue rather than of the blood Indirect evidence for the former is afforded by a study of the tissue P_{O_2} in anemic hypoxia (Aperia *et al*, 1968), where the P_{O_2} of all parts of the kidney was shown to be lower than the arterial or venous P_{O_2} which was in the normal range Nevertheless, it must be kept in mind that as long as it is not possible to determine P_{O_2} at the cellular level conveniently, determination of tissue oxygen tension will only represent an approximation

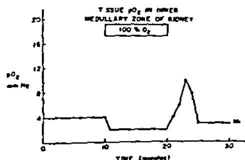


Fig 2 Continuous recording of tissue P_{O_2} during room air, and 100 % oxygen breathing (horizontal bar) Electrode position in the center of the inner medulla

In comparing renal tissue and urine P_{O_2} two facts must be considered. Firstly, the urine oxygen will be equilibrating with that of the surrounding tissue during its entire passage. It may be expected that the final P_{O_2} of the urine as it leaves the kidney is influenced particularly by the P_{O_2} of the distal portion. Secondly, rapid changes in P_{O_2} would be more easily detected in a fluid medium such as urine, in contrast with tissue where O_2 molecules would be expected to diffuse less rapidly. This is one explanation of why the "rebound response" of urine P_{O_2} upon altering the arterial P_{O_2} in the dog (Aperia and Liebow 1964) is only rarely observed in recording P_{O_2} of the outer medulla. Except for the frequent absence of rebound response in the tissue P_{O_2}, the P_{O_2} of the outer medulla and of the urine correlate well. This observation confirms the hypothesis that urine P_{O_2} sampled from the ureter during a fairly rapid diuresis reflects renal medullary tissue P_{O_2}.

Leonhardt and Landes (1963), in contrast with the present observations in the dog, found in man that P_{O_2} of urine obtained from the renal pelvis increased when 100 % O_2 rather than air was breathed. No immediate explanation can be given for this difference except that of species or possibly technique.

In good agreement with the findings of Aukland and Krog (1960) and of Ulfendahl (1962), and of Leonhardt and Landes (1963) in man is the continuous decline in renal tissue P_{O_2} from the cortex to the inner medulla. This indicates that oxygen transport is affected by a countercurrent system as is that of certain other solutes (Ullrich *et al.* 1961).

The renal cortical tissue P_{O_2} of 47 ± 18 mm Hg found in normal dogs during respiration of room air correlates well with the P_{O_2} of other tissues such as the muscle, skin and heart, recorded under similar conditions (Campbell 1928, Horwitz *et al.* 1950, Montgomery 1957, Bartlett and Tenney 1963). It should be stated that during respiration of air with the animal under Nembutal anesthesia the cortical tissue P_{O_2} is slightly lower than the P_{O_2} reported for renal venous blood. The significance of this finding with regard to the possibility of intrarenal blood or oxygen shunting, however, cannot be determined until a more sensitive and accurate method of determining tissue P_{O_2} becomes available. With the super-saturation of arterial blood when the respiratory gas is 100 % oxygen there is a constant, striking and immediate rise of renal cortical P_{O_2} (Fig. 2) that levels off at a value averaging 130 mm Hg. This response is again in good correlation with that reported for other tissues. It is noteworthy that cortical tissue P_{O_2} at this level of arterial P_{O_2} (about 500 mm Hg), exceeds the P_{O_2} corresponding to full oxygen saturation of hemoglobin. This in turn indicates that the tissue oxygen extraction will depend more or less completely on oxygen dissolved in plasma.

The specific relationship between arterial and tissue P_{O_2} of the outer medulla is of particular interest. It should be emphasized that during respiration of room air the tissue P_{O_2} of the outer medulla is only slightly lower on the average than that of the cortex, or in individual readings actually higher. This is in harmony with other evidence that the countercurrent effect and intrarenal plasma skimming occur mainly deeper in the medulla.

The fact that when the animal breathes 100 % oxygen, the P_{O_2} of the outer medulla in contrast with that of the cortex remains unchanged or actually declines as does that of the urine, is consonant with the postulated intrarenal homeostatic mechanism Aukland (1962) in his study on oxygen saturation of hemoglobin in the dog kidney observed the failure of increasing arterial P_{O_2} to raise the oxygen content of the blood in the medulla He offered the explanation that, in consequence of the S shaped oxygen dissociation curve, oxygen breathing will lead to a proportionately greater increase in arterial than in venous P_{O_2}, thereby also increasing the transfer of oxygen within the countercurrent system It is likely that this effect is of relatively small signifiacnce for the outer medulla since in the present observations the P_{O_2} of this region was but little affected by the countercurrent system Even if the increased efficiency of the countercurrent system contributed to a reduction in the response of outer medullary tissue P_{O_2} in the response of outer medullary tissue P_{O_2} to a rise in arterial P_{O_2} it could not explain an actual fall in tissue P_{O_2} following 100 % oxygen breathing Such a response can only be caused by an imbalance in the relationship between oxygen supply and oxygen consumption The possibility that arterial P_{O_2} could immediately affect medullary oxygen consumption seems unlikely for reasons previously advanced (Aperia 1964) The best explanation therefore is that arterial P_{O_2} is a controlling factor in intrarenal blood distribution, and this constitutes further indirect evidence of the existence of an intrarenal homeostatic mechanism The divergent response of cortical and outer medullary tissue P_{O_2} to changes in arterial P_{O_2} points to the vasculature of cortex or outer medulla as the primary site of action for such a homeostatic mechanism

The tissue P_{O_2} of the inner medulla differs from that of the cortex and most other tissues in two remarkable ways its low values under normal conditions, and its unresponsiveness even to extreme variations in arterial P_{O_2} The short circuiting of oxygen from the proximal to the distal loops of the vasa recta accomplished by the countercurrent system would explain the basically low tissue P_{O_2}, while the homeostatic mechanism would further protect the system against an increased oxygen input Of particular interest are metabolic studies made both *in vitro* (Gyorgy *et al* 1928, Dickens and Weil Malherbe 1936, Ullrich *et al* 1961, Kean *et al* 1962, Lee *et al* 1962, Davies 1964, Winters 1964) and *in vivo* (Herms and Malvin 1963), that suggest that the inner medulla relies mainly on anaerobic pathways for energy needs The renal medulla also seems to be well adjusted to an anaerobic system (Kean *et al* 1962) as it has been demonstrated that the mitochondria of the inner medulla have comparatively meager contents of most of the various respiratory pigments involved in electron transport It seems likely that the functioning of such a system would be abetted by a low oxygen tension (Davies 1964) The proposed intrarenal homeostatic mechanism sensitive to changes in arterial P_{O_2} would therefore be of importance in maintaining optimal function of the renal medulla

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Tubular Sodium Reabsorption and the Regulation of Renal Hemodynamics. The Effect of Chlorothiazide on Renal Vascular Resistance

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Abstract

APERIA, A C *Tubular sodium reabsorption and the regulation of renal hemodynamics The effect of chlorothiazide on renal vascular resistance* Acta physiol scand 1969 75 360—369

In hydropenic dogs under Nembutal anesthesia chlorothiazide in parallell with its inhibition of distal tubular Na reabsorption caused a marked increase in renal vascular resistance and a decrease in total renal blood flow and glomerular filtration rate The renal vasoconstriction appeared to be both pre and post glomerular, since the glomerular filtration rate was reduced in proportion to the total renal blood flow The vasoconstriction could not be abolished by sympathetic blockade (Dibenzylene) During ureteral obstruction when formation of glomerular filtrate could be regarded as minimal despite of unimpaired renal blood flow, the vasoconstrictive response to chlorothiazide was much reduced This excludes a direct constrictive effect of chlorothiazide on the renal vascular bed The constrictive response of the renal vascular bed was found to be correlated with the inhibition of Na reabsorption rather than with increase in urine flow The data are interpreted as supporting the hypothesis that Na transfer in the distal tubule is one important mechanism for controlling renal vascular tone a control that may be mediated by the renin angiotensin system It is further suggested that the important factor is the intracellular Na concentration in the first part of the distal tubule rather than intratubular concentration as such at this site Contrary to previous interpretation the present observations suggest that it is a decrease in Na transfer rather than an increase that is directly or indirectly involved

Recent studies have suggested that Na concentration in the fluid of the distal tubule may be important in the regulation of renal blood supply (Thurau 1964, Thurau and Schnermann 1965 Cortney *et al* 1966) Possibly facilitating such a mechanism is the fact that the cells of the juxtaglomerular apparatus and the macula densa are in direct apposition to each other not separated by a basement membrane The hypothesis has been advanced that a rise in Na concentration in the distal tubule would stimulate via the macula densa a juxtaglomerular release of renin. The renin would rapidly release angiotensin which would be converted into its active form and the latter would act on the renal vascular bed to cause vasoconstriction The

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reverse would apply in the case of a fall in Na concentration in the distal tubule Thurau (1964) has recently discussed the important implications of this theory, which has been proposed as an explanation of renal autoregulation The following observations have been cited in support of the validity of the renin hypothesis 1) A plasma factor, probably renin substrate, is necessary for renal autoregulation (Waugh and Shanks 1960) 2) A close relationship exists between evidence of activity of the juxtaglomerular cells and the regulation of Na metabolism (Hartroft 1963) 3) Renal autoregulation is intimately correlated with the formation of glomerular filtrate (Ochwadt 1956) 4) Decreased glomerular filtration follows the administration of certain saluretic drugs (Pitts *et al* 1958, Earley *et al* 1961)

Up to the present time incontrovertible proof has been lacking for a direct effect of distal tubular Na concentration upon renal vascular resistance Thurau's (1966) observation that retrograde perfusion of the nephron with solutions containing high Na concentrations produces vasoconstriction in individual glomeruli is suggestive This did not occur in all instances however

Material and methods

All the experiments were carried out in healthy mongrel dogs of either sex weighing 15—20 kg The animals were anesthetized with Nembutal, 30 mg per kg given intravenously, with

via the femoral artery Blood flow to the kidney was at no time interrupted since the catheter was primed and then continuously perfused with blood from a carotid artery The renal vein was catheterized under fluoroscopic guidance, using a radio-opaque polyethylene catheter (Ödman Ledin Kifa 1 or 3) with a curved tip introduced by way of the femoral or jugular vein The dye indocyanine green (0 125 mg in 0 2 ml of distilled water) was injected into a short 23 needle fitted as a sidearm of the renal artery catheter Renal vein blood was simultaneously withdrawn at a constant rate with a Harvard pump and the dye concentration of the blood was determined by means of a Gilford densitometer and Sanborn recording system Blood flow was calculated from the dye dilution curve thus obtained The use of this method has been reported previously (Aperia *et al* In press) In this system the pressure drop is 15—30 mm Hg from the aorta to the tip of the cannula an amount insufficient to affect renal blood flow A pressure drop is to be expected in part because of the relatively low resistance of the renal vascular bed. The dye method yields control values for renal blood flow within the range of those reported by clearance methods Each animal received 700 USP units of Heparin per kg body weight at the beginning of the experiment to prevent clotting in the perfusion system Blood pressure recordings from the femoral artery renal artery (by way of the sidearm of the metal catheter) and renal vein were made with Sanborn pressure transducers Renal vascular resistance was calculated as the ratio of arteriovenous pressure gradient to renal blood flow and expressed as mm Hg per ml per minute per gram kidney

samples were slowly and simultane

artery under anaerobic precautions

e method Oxygen consumption was

difference by the flow

ration specific activity 0 43 μc per mg) was used for the determination of glomerular filtration rate 0 46 mg of H^3 inulin was added to 50 ml of 10 % inulin (Warner Chilcott) Following a priming dose of 0 5 ml per kg b w the solution was given in continuous infusion of 0 1 ml per min by means of a Harvard constant rate infusion pump Standard clearance techniques were used and the urine was sampled from a ureteral catheter inserted after a retroperitoneal flank

activity of blood and urine samples was determined in a liquid scintillation counter (Packard Instrument Corporation)

The Na concentration in blood and urine samples and K concentration in urine samples were determined with a flame photometer

stal tubule (Earley
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expected to cause
control period of

30—45 min chlorothiazide (lyovac, Diuril, Merck Sharp and Dohme, 28 mg per ml in isotonic solution) was administered i.v in a priming dose of 15 mg per kg followed by continuous i v infusion at the rate of 15 mg per kg per hr

In four experiments blockade of the adrenergic receptors was obtained by the i v administration of dibenzylene (1 mg per kg) 90 min prior to the study The efficiency of the blockade was shown by the abolishment of blood pressure response to the i v injection of adrenaline (0 1—0 4 μg per kg)

In 6 expts the direct effect of chlorothiazide on the renal vascular bed was studied by temporarily stopping glomerular filtration This was accomplished by injecting 0 9 % NaCl into the ureter through an occlusive catheter until a pressure of 60—70 mm Hg was reached The ureteral catheter was connected to a Sanborn pressure transducer and the pressure was continuously recorded

Results

Chlorothiazide experiments Each animal was observed 30—45 minutes before the administration of chlorothiazide During this time glomerular filtration rate and Na reabsorption were determined during at least two urine collection periods Renal blood flow and renal vascular resistance were recorded repeatedly In most animals a determination of renal oxygen consumption was made Any animal with unstable values during the control period was not used for further studies The mean control values observed in all the animals are summarized in Table I and II The comparatively large standard error of the mean in the glomerular filtration rate determinations can be ascribed to unsatisfactory urine sampling consequent to the low flow but the use of water or osmotic diuresis would have complicated the interpretation

TABLE I Renal Function Control Data

	n	MEAN	S E M
Renal Blood Flow ml/min/100 g kidney	12	445	±27
Renal Vascular Resistance mm Hg/ml/min/g	12	26 1	± 1 83
Glomerular Filtration Rate ml/min/100 g	12	32 1	± 3 4
Glomerular Filtration Rate % renal blood flow	12	7 0	± 0 94
Urine flow ml/min/100 g	12	4 37	± 0 3
C_{Na}/C_{In} %	12	1 61	± 0 61

TABLE II Renal Function after Chlorothiazide Administration

	n	Control	After Priming Dose of Chlorothiazide	P
Renal Blood Flow ml/min/100 g kidney	7	404±78	293±78*	0 05 >P> 0 02
Renal A V O_2 diff ml/100 ml blood	7	1 69±0 41	1 75±0 31	P> 0 90
Renal Oxygen Consumption ml/min/100 g kidney	7	6 49±0 70	5 16±1 49*	0 05 >P> 0 02
Oxygen Consumed/Eq Na Reabsorbed ml/min	7	1 54±0 58	1 55±0 77	P> 0 90

*Statistically significant difference

of the data obtained The variation naturally was greatly reduced with the administration of chlorothiazide and the resulting 5 to 10 fold increase in urine flow The error in the glomerular filtration rate determinations is probably also responsible for the variations of the GFR/RBF and O consumed/Na reabsorpted ratios (Table II)

Fig 1 illustrates a typical experiment in which chlorothiazide was administered to a normal dog All of the data on the effect of chlorothiazide on renal vascular resistance glomerular filtration rate and arterial blood pressure have been summarized in Fig 2 Following the administration of chlorothiazide there was a marked and rapid rise in both Na excretion and urine flow In addition a slight increase in K excretion was generally noted During the first 15 min following the priming dose of chlorothiazide renal blood flow fell and renal vascular resistance increased gradually to a value by 45 % that of the control period The effect was consistent and statistically significant (P<0 01 for the increase in renal vascular resistance at 15 minutes) The experiments were interrupted approximately 60 min following the priming dose of chlorothiazide in order to avoid deterioration of the preparation After the first 15 min there was slight if any further increase in renal vascular the increase in renal vascular resistance The ratio of glomerular filtration rate to renal blood flow (Fig 3) however was fairly stable in the beginning of the experiment and the rise recorded after 60 min was not statistically significant (P> 0 90) No effect of chlorothiazide on systemic blood pressure was noted

In seven of the animals renal oxygen consumption was determined during the control period and 45—60 min following the priming dose of chlorothiazide (Table II) Following the administration of this substance there was a significant fall in renal oxygen consumption (P<0 05) Since the renal arteriovenous oxygen difference was practically unchanged (P>0 90) the decline in oxygen consumption

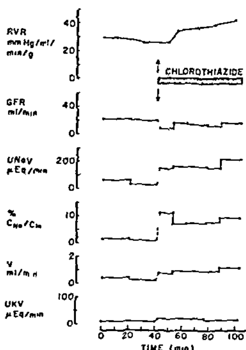


Fig 1

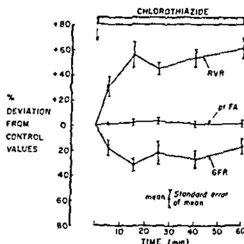


Fig 2

Fig 1 Renal response to chlorothiadize in the dog All data refer to one kidney Symbols RVR — renal vascular resistance GFR — glomerular filtration rate UNaV — urinary Na excretion, C_{Na}/C_{In} — urinary Na excretion in percentage of filtered load, V — urine flow UKV — urinary K excretion

Fig 2 Mean response to chlorothiazide of renal vascular resistance (RVR), femoral artery pressure (pr FA) and glomerular filtration rate (GFR) in 12 dogs

could be attributed entirely to the fall in renal blood flow The drop in renal oxygen consumption paralleled the fall in Na reabsorption resulting from chlorothiazide blockade

Dibenzylene and chlorothiazide experiments In four animals the effect of sympathetic block on renal vascular response to chlorothiazide was determined Dibenzylene (phenoxybenzamine hydrochloride) was administered at least 90 min prior to the control determinations Following Dibenzylene the mean systemic blood pressure fell for about 20 min and then remained constant at a level 30—50 mm Hg lower than the initial value Prior to the Dibenzylene control determinations this constant level was maintained for at least one hour during which time the pressor response to epinephrine (0 1—0 4 μg per kg) was abolished A typical Dibenzylene experiment is illustrated in Fig 4 Following administration of Dibenzylene there was a consistent fall in total renal blood flow and glomerular filtration rate This fall can probably be attributed to the lowered perfusion pressure since the renal vascular resistance also decreased After the chlorothiazide a marked increase in renal vascular

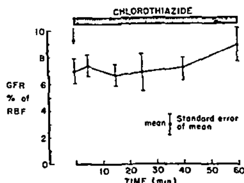


Fig 3 Mean deviations of the filtration of total renal blood flow following chlorothiazide administration in 12 dogs

resistance was observed in all Dibenzylene experiments A further reduction of the glomerular filtration rate and total renal blood flow accompanied the increase in renal vascular resistance No specific effect of chlorothiazide on the systemic blood pressure was noted after Dibenzylene

Ureter occlusion and chlorothiazide experiments In six animals temporary cessation of glomerular filtration was obtained by raising ureteral pressure to approximately 60 mm Hg Data from a typical experiment are recorded in Fig 5 The effect of chlorothiazide on renal vascular resistance in all experiments has been sum-

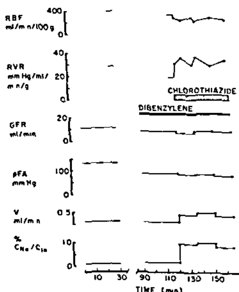


Fig 4

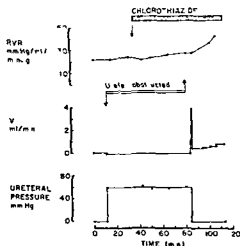


Fig 5

Fig 4 Renal vascular response to chlorothiazide following sympathetic block with Dibenzylene Symbols RBF — renal blood flow, other symbols as in Fig 1

Fig 5 Renal response to chlorothiazide in dog with obstructed ureter All data refer to one kidney Symbols As in Fig 1

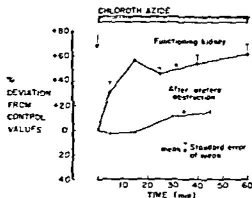


Fig 6 Renal vascular response to chlorothiazide in kidneys with ureter obstruction (6 experiments) as compared to the response in filtering kidneys.

marized in Fig 6 The total renal blood flow and renal vascular resistance were generally not affected by the ureteral obstruction. In two animals a slight fall in renal vascular resistance was noted. A significant increase in renal vascular resistance was never observed following ureteral obstruction. The hemodynamic response to chlorothiazide was found to differ markedly in the nonfiltering kidney as compared with the normal. During the first 20—30 min following the priming dose of chlorothiazide renal vascular resistance was unaltered After 30 min a slight increase in renal vascular resistance was sometimes noted. Following release of the ureteral obstruction the production of urine started almost immediately (Fig 5) Since the animals were still receiving chlorothiazide, urine flow and Na excretion were much higher than during the control period. It should be noted that following reinstitution of the filtration process renal vascular resistance rose promptly to the high level observed when chlorothiazide was given to an animal with filtering kidneys.

Discussion

It has previously been reported by Gillenwater *et al* (1962) that chlorothiazide has no effect on renal vascular resistance These investigators used an isolated perfused kidney and the perfusion rate was in the range of 53 ml per min per 100 g approximately one-eight the normal value No attempt was made to determine urine flow It is well documented that the blood flow to the normal kidney is in the range of 350—450 ml per minute per 100 g Bard 1961) Thus at a flow rate of 53 ml per minute per 100 g renal function should be greatly impaired and cessation of glomerular filtration is to be expected. The study by Gillenwater *et al* (1962 is thus comparable to the ureteral obstruction experiments

The present experiments on the nonfiltering kidney demonstrate that chlorothiazide has little direct effect on the renal vascular bed. The increase in vascular resistance of the filtering kidney following chlorothiazide administration must therefore be related to some effect of the drug associated with urine formation Statistical analysis suggests that this vasoconstrictive effect is related to the block in Na reabsorption rather than to increase in urine flow (Fig 7 and 8) The correlation co-

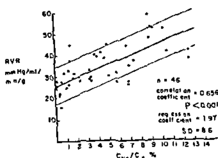


Fig 7

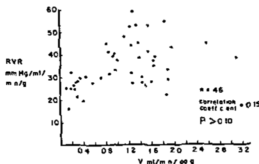


Fig 8

Fig 7 The relationship between the excretion of Na as percentage of filtered load (C_{Na}/C_{In}%) and renal vascular resistance

Fig 8 The relationship between urine flow and renal vascular resistance

efficient between C_{Na}/C_{In} in renal vascular resistance was highly significant ($P<0.001$), while no correlation could be found between urine flow and renal vascular resistance ($P>0.1$) The maintenance of the vasocontrictive effect of chlorothiazide in animals pretreated with Dibenzylene demonstrates that the sympathetic system plays a minor role in this response Thus the data presented strongly support the hypothesis that Na concentration and/or transport in the distal tubule regulates renal vascular tone and renal blood supply via a pressor mechanism, or a substance that is not a catecholamine

The data from the present study are insufficient to define the precise mechanism or pressor substance Previous studies, however, suggest the involvement of the renin-angiotension system If it is assumed that the renal vasoconstrictive effect is due to an increased action of angiotensin, the question arises why it should act only on the renal vascular bed, no effect on the systemic blood pressure was noted This observation is in accord with clinical experience with chlorothiazide in normotensive subjects (Hollander *et al* 1959) The lack of systemic response might be explained by the following hypothesis The renin activated angiotensin acts in the first instance locally on the renal vascular bed, presumably the afferent arteries Under certain conditions, however, such as constriction of the renal artery outside of the kidney, it has been observed that the renal vascular bed is less sensitive to angiotensin-II (McGiff and Itskovitz 1964) In such circumstances the kidneys might be considered to be more easily saturated with angiotensin This would result in the increased renal venous outflow of pressor substance that is well established following renal artery constriction (Kohlstaedt and Page 1940, Skinner *et al* 1963)

Another unknown factor is the mechanism by which the Na concentration in the distal tubular fluid could trigger a release of renin The present study has demonstrated a direct relationship between the block in Na reabsorption and the degree of renal vascular constriction (Fig 7) The data do not, however, allow a distinction between the effect of the blockade of Na transport as such and the effect of the

probable increase in Na concentration of distal tubular fluid Certain observations support the first hypothesis rather than the latter as presented by Thurau (1964) It is generally accepted that the macula densa cells, by enzymatic action activate the production of renin Whether renin is actually produced in the juxtaglomerular apparatus or in the macula densa and only stored in and released from the juxta glomerular cells is still open to question (Bing 1963, Hartroft *et al* 1964) There is substantial evidence however that the enzymatic activity of the macula densa cells is closely related to the production of renin (Fisher 1961 Hess 1963) The macula densa enzymes involved in this process are reported to be inhibited by increase in Na concentration (Reeves 1965) Blockade of Na reabsorption in the distal tubule would tend to lower the intracellular Na concentration at this site Thus the enzymatic activity of the macula densa cells would be enhanced with a consequent increase in renin production An example of the opposite effect is the vasodilatation produced by a mannitol load (Lilien *et al* 1963 Detmer *et al* 1965) which could be explained on the basis of increased Na concentration in the macula densa cells Mannitol is known to inhibit proximal tubular reabsorption of Na thereby making more Na available to the distal tubule Since free water formation during water diuresis is increased by mannitol load distal tubular Na reabsorption must be enhanced under this condition Thus Na concentration of the macula densa cells will increase resulting in depression of enzymatic activity and inhibition of renin production The proposed mechanism is opposite to that proposed by Thurau (1966) and in part supported by his recent experiments Further actual measurements of Na concentration and transport in the distal tubule are needed to resolve the question of what mechanism if any involves this ion in the regulation of renal vascular tone

The consequence of the apparently Na related renal vasoconstriction is a decrease in renal blood flow and O consumption Under physiologic conditions thi is an energy conserving process since the reabsorption of Na accounts for the major part of the renal oxygen consumption (Lassen *et al* 1961 Kiil *et al* 1961) In the present experiments O consumption was indeed reduced concomitantly with a fall in Na reabsorption Since the renal arteriovenous O difference was constant before and after chlorothiazide the fall in O consumption was closely linked to the reduction of renal blood flow The renal vasoconstriction related to the block of Na reabsorption appears to be both pre and postglomerular filtration rate was reduced in proportion to the total renal blood flow (Fig 3) The slight increase in the *GFR/RBF quotient observed towards the end of the experiment might suggest* a more pronounced effect on the post glomerular filtration rate determinations are too great to permit definite conclusions

The regulatory effect of tubular Na reabsorption on renal vascular tone appears to be related to renal metabolic control under normal conditions When Na reabsorption is disturbed by disease or drug action undesirable effects on renal hemodynamics can be expected The present study has clearly shown that chlorothiazide causes vasoconstriction in the normal kidney Although no comparable work on the hemodynamic effect of chlorothiazide in the diseased kidney is available it is possible that

there is a vasoconstrictive effect similar to that observed in the normal The use of such saluretic drugs in the presence of renal disease should therefore be reconsidered

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Tubular Sodium Reabsorption and the Regulation of Renal Hemodynamics. The Effect of Hypertonic Saline Infusion on Renal Vascular Resistance

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Abstract

APERIA A C, A A LIEBOW and L E ROBERTS *Tubular sodium reabsorption and the regulation of renal hemodynamics The effect of hypertonic saline infusion on renal vascular resistance* Acta physiol scand 1969 75 370—376

The hemodynamic response following saline loading was found to differ from that induced by the saluretic agent chlorothiazide, which causes vasoconstriction When hypertonic NaCl was infused into the renal artery until a profuse sodium diuresis was obtained and the infusion then interrupted C_{Na}/C_{In} remained elevated for 60—80 min During this period the renal vascular resistance did not change significantly and the glomerular filtration rate tended to rise as compared with the control periods This is contrary to what has previously been found during chlorothiazide administration where renal vascular resistance was significantly correlated with C_{Na}/C_{In} A hypertonic saline load blocks Na reabsorption in the proximal tubules chlorothiazide mainly in the distal tubules It was therefore concluded that it is a reduction of movement of Na into the distal tubule cells specifically the macula densa rather than the increased Na concentration of the distal tubular fluid that is responsible for the vasoconstrictive effect of chlorothiazide

It has recently been demonstrated that blockade of distal tubular Na reabsorption by chlorothiazide is associated with an increase in renal vascular resistance (Aperia, to be published) This finding was interpreted to support the hypothesis that there is a relationship between Na reabsorption and renal vascular resistance which is mediated by the renin angiotensin system Thurau (1964) postulated that an increased Na concentration in distal tubular fluid at the site of the macula densa might trigger the release of renin from the juxtaglomerular cells Others have suggested that it is the intracellular Na concentration of the macula densa that is of critical importance for the production and release of renin (Reeves and Sommers 1965), more specifically that the production of renin would be enhanced by de

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crease in intracellular Na concentration This would be contrary to the Thurau hypothesis Chlorothiazide is now thought to inhibit Na reabsorption predominantly in the relatively water impermeable cells of the distal tubule (Earley *et al* 1961) The administration of this drug could thus result in a simultaneous decrease in Na concentration in the cells of the distal tubules and an increase in tubular fluid Na concentration The vasoconstrictive effect of chlorothiazide could therefore be related to either of these factors To elucidate the relationship between Na reabsorp tion production of renin, and control of renal blood flow, a system would be needed where intracellular and tubular fluid Na concentration could be altered independently in either direction. There is good reason to believe that sodium concentration in the tubules can be raised in the case of loading with hypertonic saline Proximal tubular fluid has been shown to be isosmotic to plasma in dogs through a full range of osmotic levels Elevation of plasma Na may therefore be expected to produce an elevation in the concentration of Na in the tubular fluid as it enters the distal tubule (Dirks *et al* 1965)

In the present work the attempt was to achieve a maximal effect of saline on tubular Na transport by the infusion of hypertonic NaCl into the renal artery Relationships between renal vascular resistance and Na excretion and reabsorption were then determined The data suggest that it is the intracellular Na concentration that is of primary importance for the control of renal blood flow presumably in relation to the production of renin

Material and methods

Ten healthy mongrel dogs weighing 15—20 kg were used for this study The animals were under Nembutal anesthesia 30 mg per kg with small supplements as required and breathed air from pressure tanks via a Burns demand respirator The studies were carried out with the dogs in hydropenia The animals had been fasted for 15 hrs and received during the ex periment 0 9 % CaNl i v at a rate of 0 10—0 15 ml per min per kg

Renal blood flow was determined by a dye dilution method For this purpose the renal artery was catheterized from within the aorta by means of a large thin walled metal catheter inserted via the femoral artery Blood flow to the kidney was at no time interrupted since the catheter was continuously perfused with blood from the carotid artery The renal vein was catheterized under fluoroscopic guidance using a radiopaque polyethylene catheter with a curved tip inserted via the femoral or jugular vein The dye indocyanine green (0 125 mg in 0 2 ml of [illegible]

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animal received 700 USP units of Heparin per kg at the beginning of the experiment to prevent clotting in the perfusion system Blood pressure recordings from the femoral artery renal artery and renal vein were made with Sanborn pressure transducers Renal vascular resistance was calculated as the quotient of arteriovenous pressure gradient and renal blood flow and expressed as mm Hg ml/min/g kidney

The clearance of tritiated inulin (Baird Atomic Corporation specific activity 9 43 μc/mg) was used for the determination of glomerular filtration rate 0 46 mg of H3 inulin was added to 50 ml of 10 % inulin Warner Chilcott Following a priming dose of 0 5 ml per kg b w the solution was given in continuous i v infusion of 0 1 ml per minute Standard techniques for measurement of clearance were used and the urine was sampled from a ureteral catheter inserted retroperitoneally via a flank incision The radioactivity of blood and urine samples was determined in a liquid scintillation counter (Packard Instrument Corporation)

Na concentrations in blood and urine samples and K concentration in urine samples were determined with a flame photometer

Plasma volume was estimated from dilution of Evans blue in four of the animals The dye dilution of a plasma sample obtained 10 min following the dye injection was determined with a Beckman spectrophotometer The animal s own plasma obtained before the dye injection was used as a blank

As stated, saline loading in the dog inhibits Na reabsorption in the proximal tubules and increases distal tubular Na reabsorption (Dirks *et al* 1965) In hydropenia this is reflected in increases in free water reabsorption Na excretion and C_{Na}/C_{In} In the present study only the last two parameters have been used to estimate the effects on the tubules It was found that a maximal tubular saline effect was obtained by the infusion of 10 % NaCl into blood entering the renal artery for 5—15 min Following the infusion the plasma Na concentration was elevated and the Na excretion and C_{Na}/C_{In} remained increased for 60—80 min The 10 % *NaCl solution was introduced into the perfusion system of the renal artery at a rate of* 2 5—3 5 ml per min

Results

The protocols of two typical experiments are shown in Table I and II

1 *Effect on plasma Na concentration and body compartments*

During NaCl *infusion* Na concentration of blood in the renal artery was in the range of 195—205 meq/l The increase in plasma Na concentration following the infusion in expt 1 Table I is representative of all experiments From these data it can be calculated that the Na supplied was rapidly and equally distributed The total increase in body Na after infusion was 116 meq—12 meq Na excreted The plasma Na constitutes 15 8 % of the total exchangeable Na (Edelman and Liebman 1959) Assuming an even Na distribution without changes in plasma volume the increase in plasma Na concentration would then be $\frac{15\ 8 \times 104}{100 \times 1\ 17}$ meq/l = 13 9 meq/l which is not far from the observed value of 13 0 meq/l The plasma volume was measured in four of the animals towards the end of the experiment It was found to be in the normal range 4 5—5 2 % of the b w

2 *Effect on urine flow Na excretion and reabsorption and K excretion*

Urine flow and Na excretion increased almost instantaneously during infusion of hypertonic NaCl into the renal artery Urinary Na concentration increased 5—20 fold The reabsorbed fraction of the filtered Na decreased This occurred in all experiments including those in which the glomerular filtration rate during the NaCl infusion was reduced to the extent that the amount of filtered Na remained practically unchanged Thus the blockade of Na reabsorption is related to the increase in Na concentration of the filtered fluid The animals were observed for 40—80 min following the termination of the NaCl infusion. During this period the plasma Na did not fall significantly This is to be expected since total urinary Na excretion during this period ranged from 3—7 % of the Na infused Although the plasma Na concentration was fairly stable following the infusion C_{Na}/C_{In} slowly declined and in some cases approached control values after 70 min Urinary K excretion generally increased 2—3 fold during the NaCl infusion and remained high as long as C_{Na}/C_{In} was elevated It has already been shown by Suki *et al* (1965) that the relationship

TABLE I

	Time min	PNa meq/l	V ml/min	UNaV µeq/min	C_{Na}/C_{In} %	UKV µeq/min	GFR ml/min/100 g kidney	RVR mm Hg/ml/min/g kidney
Control Period	—30—0	146	0 06	4 3	0 15	18 0	20 0	21 0
Infusion Period[1]	0—17	157 (209)[2]	2 40	646	8 23	45 2	38 6	
	7							34 7
	12							33 1
	14							30 6
Post Infusion Period	24—32	156	0 55	155	2 94	24 4	33 3	19 6
	32—42	156	0 40	101	2 14	25 1	30 4	21 8
	42—55	157	0 29	91	1 60	35 0	32 1	26 2
	55—71	157	0 22	52	1 33	32 0	24 3	28 0

Body Wt = 22 7 kg
Plasma Volume towards end of experiment = 1 17 l

[1] Total amount infused = 68 cc = 116 mEq Na
[2] Na concentration of renal artery blood

between urinary K and Na excretion is the same during saline loading as during chlorothiazide administration The increase in K excretion is interpreted as the consequence of increased Na delivery to the Na^{+}—K^{+} exchange site in the far part of *the distal* tubule

3 *Effect on glomerular filtration rate, renal blood flow and renal vascular resistance*
The glomerular filtration rate changed inconstantly upon infusion of NaCl In 3 of 12 expts it increased and in 4 of 12 expts it decreased during the infusion Following the infusion the glomerular filtration rate was elevated in 5 of 12 expts In only two of 12 expts a moderate reduction of the glomerular filtration rate was noted following the infusion (19 % and 21 % of the control values) The total renal blood flow generally decreased and the renal vascular resistance increased during NaCl infusion This effect was often temporary, however, and subsided during the continued infusion Since vasoconstriction was also noted when hypertonic NaCl was infused into the mesenteric artery during these experiments this was interpreted as a direct effect of the NaCl in high concentration on the vessel wall Following termination of the infusion urinary Na excretion and C_{Na}/C_{In} remained elevated,

TABLE II

	Time min	PNa meq/l	V ml/min	UNaV μeq/min	C_{Na}/C_{In} %	UKV μeq/min	GFR ml/min/ 100 g kidney	RVR mm Hg/ ml/min/g kidney
Control Period[1]	—30—0	150	0 05	0 92	0 07	11 3	9 4	32 1
Infusion Period	0—16	158 (194)*	1 13	308	14 0	56	6 5	43 4
Post	20—32	164	0 36	76	5 01	38	9 2	38 1
Infusion	32—45	165	0 13	21	1 34	44	10 0	38 1
Period	45—67	166	0 08	7 7	0 39	33	12 1	36 3

but renal vascular resistance always returned to normal Thus no correlation could be established between renal vascular resistance and C_{Na}/C_{In} following hypertonic saline load (Fig 1) This is contrary to the findings following chlorothiazide administration The systemic arterial pressure was unchanged during and following the infusion.

Discussion

Although a statistically significant correlation between renal vascular resistance and C_{Na}/C_{In} was demonstrated following chlorothiazide administration (Aperia to be published) these experiments did not permit a precise definition of such tubular factors as might be involved in the production and release of renin Such a system may be conceived to constitute a possible means of intrarenal hemodynamic control Following hypertonic saline loading urinary sodium excretion and C_{Na}/C_{In} were also

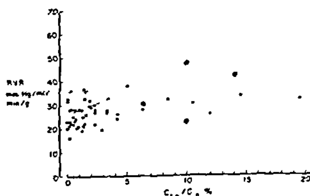


Fig 1 The relationship between C_{Na}/C_{In} and renal vascular resistance following the infusion of 10 % NaCl into the renal artery No statistically significant correlation could be demonstrated Broken line represents the correlation between C_{Na}/C_{In} and renal vascular resistance during chlorothiazide administration Encircled dots refer to values obtained during the infusion of the NaCl

found to be elevated but not significant effect on renal vascular resistance could be demonstrated A comparison of the effects of chlorothiazide and saline loading may provide a clue to the mechanisms of intrarenal hemodynamic control

Chlorothiazide and saline administration have the same secondary effect on K excretion Their diverse effect on renal vascular resistance will therefore rule out K transport as a primary factor in intrarenal hemodynamic control This is in accord with the finding that the production of renin as inferred from histological observations can be correlated with the metabolism of Na but not with the metabolism of K (Hartroft 1963)

Chlorothiazide and loading with hypertonic saline both result in an increase in the sodium concentration of the fluid of the distal tubules This cannot be responsible for the vasoconstrictor effect observed with chlorothiazide since it does not occur with saline loading The effects of chlorothiazide and saline loading however differ with regard to the sodium concentration in the cells of the distal tubule With sodium loading it may be inferred that distal tubular reabsorption of Na is enhanced in the dog (Dirks *et al* 1965) although the precise localization in the distal tubule is not known With chlorothiazide sodium transport is inhibited in the first part of the distal tubule and thus presumably also in the macula densa region. A reduction of the intracellular sodium concentration of the macula densa can therefore be expected. This decreased distal tubular sodium transport appears to be the only known factor related to the vasoconstrictor effect of chlorothiazide

The hypothesis that it is the intracellular sodium concentration in the macula densa that is the major factor in the control of production and relase of renin is supported by a number of observations both in humans (Brown *et al* 1964) and in animals (Hartroft and Hartroft 1953 Tobian 1960) It has been observed that renin production is decreased when the diet is high in sodium and increased when the diet is low in sodium

Variations in distal tubule Na transport and intracellular Na concentration can also explain the renal autoregulation phenomenon. There is already substantial evidence that suggest that he renal vasoconstriction produced by elevated perfusion pressure is mediated via the renin angiotensin system In experiments on dogs elevation of the renal perfusion pressure causes natriuresis and impairment of the concentration capacity Selkurt *et al* 1965) In humans with essential hypertension it has been demonstrated that free water formation during water diuresis is decreased (Steinmetz 1961) Thus distal tubular Na transport should be lowered creating a condition quite similar to that during chlorothiazide administration

Since Na reabsorption is the major determinant of renal oxygen consumption (Lassen *et al* 1961 Kiil *et al* 1961) it might seem an attractive hypothesis that with an increase in filtered Na a higher Na concentration in distal tubular fluid would produce renal vasoconstriction via the reninangiotensin system thus lowering the work load and the need for oxygen. In the case of increased filtered Na load due to increased Na concentration of the filtrate this hypothesis can be discarded In stead the vicious cycle of increased work load increased oxygen need and the need

for further blood supply which would produce concomitant increase in glomerular filtration rate, is probably prevented by the existence of a factor limiting proximal sodium reabsorption.

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From the Department of Physiology, University of Göteborg, Sweden

Neurogenic Adjustments of Muscle Blood Flow, Cutaneous A-V Shunt Flow and of Venous Tone during "Diving" in Ducks

By

A. M. Djojosugito[1], B. Folkow and L. R. Yonce[2]

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Abstract

Djojosugito, A. M., B. Folkow and L. R. Yonce. *Neurogenic adjustments of muscle blood flow, cutaneous A—V shunt flow and of venous tone during "diving" in ducks.* Acta physiol. scand. 1969. *75.* 377—386.

The effect of "diving" on the vascular response in skeletal muscle, the web and the veins was studied in experiments on ducks. Muscle blood flow completely stopped during submersion due to neurogenic vasoconstriction of the resistance vessels, at the same time as the constrictor fibres to the systemic veins were excited. As a contrast, web blood flow changed only little during submersion, unless the duck was excited. In this latter case it decreased, whether the animal was submerged or not. The profound muscle vasoconstriction helps to conserve the oxygen stores for the "essential" tissues, the heart and the brain. The preserved web blood flow, on the other hand, primarily passes through A—V shunts and, in conjunction with the venous constriction, it pushes blood from the considerable, oxygen-containing venous depots to the heart without causing peripheral oxygen "losses" from the blood. Such mechanisms serve to make the total venous oxygen stores available for subsequent cardiac output to the brain and the myocardium.

Cardiovascular responses during submersion in most diving animals include bradycardia and increased peripheral resistance with little change in arterial blood pressure. The distribution of the decreased cardiac output, associated with the bradycardia, seems to be organized to deliver the oxygen stores in blood and lungs preferentially to vitally important tissues as the brain and myocardium. However, to be really efficient the "diving response" presupposes a most profound reduction of blood supply to other tissues, since they would otherwise consume considerable fractions of the oxygen stores. The literature on the cardiovascular adjustments to diving was recently reviewed by Andersen (1966) and included studies that have evaluated the blood flow to various tissues during the diving response.

The large increase in lactic acid concentration of the arterial blood during the

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immediate post-dive period in seals strongly suggests that the blood flow to skeletal muscles is very limited during submersion (Scholander 1940). Further, Grinnel, Irving and Scholander (1942) and Andersen (1959), using thermocouple devices found evidence of a markedly decreased blood flow through the skeletal muscles during a dive, though the quantitation of this type of measurement is difficult to assess.

Hollenberg and Uvnäs (1963) observed in ducks that cutaneous blood flow in the wing, usually measured in the divided brachial artery, was considerably decreased while intestinal blood flow, measured as the venous effluent, could virtually stop during submersion. However, when they recorded muscle blood flow in the leg by means of a drop recorder inserted into the femoral (sciatic) artery, the vasoconstriction, which in their preparations seemed to be relatively pronounced initially, was usually not further intensified during the dive. Johansen (1964), on the other hand, utilizing the regional distribution of Rb^{86} in the duck during diving, found that very little isotope reached the skeletal muscles. The same was true for the gastrointestinal tract and for most parts of the skin.

These somewhat controversial and partly qualitative data require additional experiments to clarify the extent of the vascular adjustments during diving in the skeletal muscles and other major vascular beds, where vasoconstriction may be of particular importance for the conservation of the oxygen stores for the essential tissues, the brain and the heart. The purpose of the present study was to determine in ducks the effect of submersion on muscle blood flow, and also on blood flow through the cutaneous A-V anastomoses. These cutaneous A-V anastomoses in typical parts of the body may provide the "shunt flow" which seems to be needed for efficient turnover of all the oxygen-carrying venous depots to the heart for subsequent output to the brain and myocardium. In addition, the participation of the systemic veins in the neurogenic vasoconstrictor response during submersion was explored. A preliminary report of part of the present results has recently been published (Folkow, Nilsson and Yonce 1966, Folkow 1966).

Methods

Experiments were performed on 20 ducks, which were lightly anesthetized with a short acting barbiturate (Narcotal, Astra) during such parts of the preparation that might inflict pain. In all animals arterial blood pressure and heart rate were continuously measured from one of the brachial arteries on a Grass Polygraph.

Blood flow through the web. To study the responses of the cutaneous A-V anastomoses the web was chosen since this tissue seems to contain a great number of these specialized vessels normally involved in the temperature regulation. After heparinizing the duck, web blood flow was measured with a drop recorder connected to a cannula in the main vein draining the web. This cannula was inserted through a small incision in the "horny" tissue just above the web during short-term anesthesia. Great caution was taken not to damage either the arterial or nervous supply. The ducks were suspended in a sling with the cannulated web hanging through a hole. When habituated [illegible] ed quietly and did not appear to have any discomfort from the [illegible] through the drop [illegible] from the web was [illegible] to [illegible] be a cannula in [illegible] by [illegible] The content of [illegible] el with an [illegible] venous [illegible] fore during [illegible]

2 Muscle blood flow Cannulation of the femoral vein, draining the entire limb necessitates a large operation and cannot be performed under local anesthesia, since the anesthetic would affect also the vasoconstrictor fibre control of the vascular bed. Further there are considerable vascular anastomoses in the thigh, allowing extensive by pass of blood, which may give erroneous figures if flow is measured only in the major artery or vein without anastomotic obstruction. It was therefore considered necessary to isolate a well-defined muscle region in such a way that its entire venous outflow could be recorded. Moreover, vasoconstriction of also large arteries seems to constribute markedly to the neurogenic flow reduction in diving species (Folkow Fuxe and Sonnenschein 1966) It was considered important to leave not only the sciatic nerve but also the femoral artery entirely intact, since a major part of the constrictor fibre supply to larger arterial branches appears to run along its walls

General anesthesia abolishes the diving response, while decerebration leaves the response essentially intact (Andersen 1966) Therefore, the ducks, in which total calf blood flow was to be measured were decerebrated either by means of electrocautery through the brain stem at the posterior hypothalamic region, or by means of extirpation of the entire brain cranially to the posterior end of the optic lobes Then the main vessels and nerves of the limb were approached from the dorsal side and all connections to the calf muscles except the femoral vein the femoral artery and the sciatic nerve, were divided between ligatures After heparinization a drop recorder was inserted between the central and peripheral ends of the femoral veins for continuous measurement of the venous effluent from the calf In the course of the experiment the web could be tied off and the calf muscles skinned so as to leave only the muscle blood flow Blood flow, blood pressure and heart rate were recorded on a Grass Polygraph.

3 Recordings of venous responses In these experiments the same isolation of the calf muscles was performed and blood flow was measured in the same way, but now with an "open drop recorder unit so that venous outflow pressure could be set at any desired level. In addition the calf was inserted into a water filled plethysmograph, using the skin as a water tight seal in the same way as has earlier been described for the cat's calf muscles (Kjellmer 1964) Volume changes of the calf, thus reflecting preferentially changes in regional blood content, were recorded by means of a sensitive piston recorder, writing on a smoked drum. Sometimes both artery and vein were temporarily obstructed and the occurrence of neurogenic venoconstriction during submersion could then be recorded "isometrically" as an increase in venous pressure within the calf preparation —In a few cases the blood supply to the wing was interrupted by a rubber cuff, while venous pressure within the obstructed vascular compartment was continuously recorded Neurogenic venoconstriction was here revealed as a rise in venous pressure which could be abolished by local anesthesia blockade of the regional nerves

Results

1 Web Blood Flow

The vascular bed of the web like that of the pads of most mammals, seems to be dominated by cutaneous A—V anastomoses normally involved in the centrally directed heat loss control. It is also well known for most species that intense but phasic constrictions of the blood vessels in such cutaneous areas are often induced when apprehension fear or rage is induced

It also soon became evident in the duck that web blood flow was markedly involved in such centrally elicited responses via their vasoconstrictor fibres Thus touching the duck or mere movements towards the animal especially during the early phase of the experiment would often cause prompt but transient decreases of web blood flow and tachycardia However, when the animals were gently handled and became habituated to the procedures the web blood flow was less and less affected by such events Further, the strong involvement of the web vessels in the heat loss control was evident by the prompt increase of web blood flow when a small amount of warm Tyrode solution was intravenously injected while cool solutions produced a flow decrease

immediate post-dive period in seals strongly suggests that the blood flow to skeletal muscles is very limited during submersion (Scholander 1940). Further, Grinnel, Irving and Scholander (1942) and Andersen (1959), using thermocouple devices, found evidence of a markedly decreased blood flow through the skeletal muscles during a dive, though the quantitation of this type of measurement is difficult to assess.

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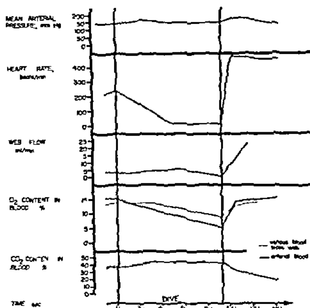


Fig 2 Unanesthetized duck, 2 8 kg Diagram illustrating the changes in arterial blood pressure heart rate web blood flow oxygen and carbon dioxide contents in arterial blood and in the venous effluent from the web before during and immediately after submersion

to protest by intense struggling virtually stopped web blood flow while the diving bradycardia was quite feeble and often interrupted by bouts of tachycardia (upper section) The lower section of Fig 1 was obtained about 90 min later when the animal by gentle handling had become habituated to the brief submersion periods and was so calm that it even accepted food Resting heart rate was considerably reduced Further, during submersion of the head the animal remained calm and now this procedure produced intense and regular bradycardia which was no longer accompanied by any significant reduction of web blood flow Upon emersion the web blood flow increased phasically above the control value then returned to the initial level essentially in pace with the elimination of the post dive tachycardia and hypertension This transient rise in web blood flow seemed to be a matter predominantly of the raised perfusion pressure during the post dive period since there was little change of regional flow resistance

To explore whether the maintained web blood flow during submersion predominantly reflected flow through non metabolic vascular pathways *i e* through cutaneous A—V shunts the O_2 and CO_2 contents of the arterial blood and the venous effluent from the web were measured in some of the experiments Such an experiment is shown in Fig 2 It is clear from this figure that no significant uptake of O_2 from the blood or delivery of CO_2 to the blood occurred in the well perfused web during submersion If anything the O_2 content of the venous effluent was higher than that of the arterial blood which was sampled from the aorta via a brachial artery The probable reason for this apparent paradox is that simultaneous samplings of blood at these points of the vascular bed implies that the arterial

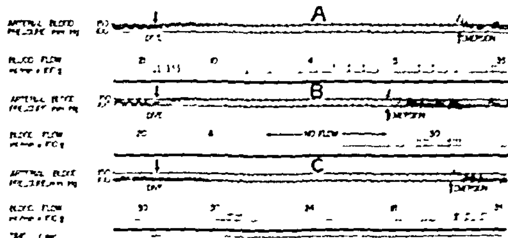


Fig. 3. Decerebrated duck, 3.1 kg. Effects of submersion on arterial blood pressure, heart rate and blood flow in the right leg.

In section A the circulation in the skin and web were included in the flow recording.

In section B only muscle blood flow was recorded since the web had been excluded and the calf muscle skinned. Note the complete cessation of muscle blood flow during the peak of the diving response.

In section C the vasoconstrictor fibres to the calf muscles had been blocked. The vasoconstrictor response to submersion was now very small and was presumably due to circulating catecholamines.

sample must represent a later time stage of the submersion period than the blood which just passed the web because of the greatly reduced movement of blood in these lower parts of the body. In any case, these data strongly suggest that the by far major part of the web blood flow has passed through A–V shunt vessels.

2. *Muscle Blood Flow*

An experiment illustrating the changes in muscle blood flow during submersion is shown in Fig. 3. In section A the circulation through the web and skin still had intact connections with the calf muscle preparation. Despite a pronounced reduction of blood flow through the preparation during submersion, an appreciable blood flow remained. Between sections A and B the calf was skinned and the web was tightly ligated so that only the muscle vascular bed remained. Now submersion produced a complete cessation of blood flow, and this intense vasoconstriction in muscle developed in pace with the pronounced bradycardia response. Between sections B and C the sciatic nerve was cut and the femoral artery was soaked in xylocaine to block the vasoconstrictor fibres along its walls. Now submersion produced only a slight and retarded decrease of muscle blood flow, which might be ascribed e.g. to catecholamines secreted by the adrenal glands and carried by the blood stream.

It seems to be characteristic of decerebrated ducks that the normally sudden and intense post-dive tachycardia and blood pressure rise is far less prompt in onset and less intense than in intact animals. This is illustrated in Fig. 3, as is also the fact

that the post dive increase of muscle blood flow is then moderate in extent and relatively slow in onset In the intact animal it appears as if blood flow to peripheral tissues must be greatly and suddenly increased, as judged indirectly from the fact that huge increases in cardiac output occur during the early post dive period (Folkow, Nilsson and Yonce 1967)

3 Venous Responses

These experiments revealed that submersion produced a considerable reduction of calf blood content in connection with the huge increase in regional flow resistance This expulsion of blood was not merely a consequence of passive elastic recoil, due to the reduced transmural pressure within the venous compartment, which always seems to occur in skeletal muscles in connection with precapillary vasoconstrictions If both the femoral artery and vein were temporarily occluded just prior to the head submersion venous pressure started to rise in the occluded vascular bed of the calf If at this stage the venous clamp was selectively released, blood has forcibly expelled from the calf The same was true for the wing when its vascular bed was occluded and the regional venous pressure recorded If, on the other hand, the same procedure was repeated after xylocaine block of the regional vasomotor nerves, no such effects were obtained suggesting that a neurogenic venoconstriction forms part of the diving response

Discussion

Earlier measurements of cardiac output in ducks during submersion indicate that such marked reductions can be induced (down to 60—70 ml/min compared with resting control values of 1200—1500 ml/min in a 3 kg duck, Folkow, Nilsson and Yonce 1967) that the blood supply to several major tissues such as skeletal muscle, gastrointestinal tract kidneys most parts of the skin etc must be virtually stopped if the supply to the vitally important tissues, as the heart and CNS is to be maintained or even increased

To judge from the regional Rb^{86} distribution (Johansen 1964) there is a marked reduction in muscle skin and gastrointestinal blood flow during submersion though such a method will hardly give quantitatively correct figures due to several pitfalls inherent in the technique For example Rb^{86} extraction varying with the rate of capillary flow is usually more complete when blood supply is reduced as long as the flow passes through nutritional channels and not through shunts (*cf* Dresel Folkow and Wallentin 1966) This method will consequently overestimate flow where flow is reduced and *vice versa* while it will not reveal whether any true shunt flow occurs

The present experiments show that muscle blood flow which in the resting equilibrium in ducks is of the order of 20—40 ml/min × 100 g (see also Folkow, Fuxe and Sonnenschein 1966) can be entirely stopped by the vasoconstrictor fibre discharge during submersion These earlier experiments have further shown that

This lack of neurogenic constriction of apical cutaneous shunts may indeed be of great importance for the cardiovascular adjustments during submersion. Since the metabolic" vascular pathways of several major vascular circuits seem to be virtually ned, their venous oxygen containing blood would become largely stagnant and nce of little use for the supply of the brain and myocardium if it is not moved to- ards the heart This movement would not be efficiently accomplished by a blood eam through metabolic vascular channels, since this would also imply peripheral 2 'losses'

However, shunt blood flow through apical body sections will, without such O_2 osses', move the venous depots, which, moreover, by the reported neurogenic noconstriction will be displaced from peripheral tissues towards larger veins, back the heart for subsequent output to those vascular circuits that are not obstructed y the intense vasoconstrictor fibre discharge It is here likely that the neurogenic nptying of the most peripheral sections of the venous compartment is important y causing a "centralization' of the systemic venous depots, so that they can uantitatively be moved towards the heart by the apical shunt flow The occurrence f venoconstriction in connection with diving is further indirectly supported by the act that central venous pressure may reach very high levels during submersion Folkow, Nilsson and Yonce 1967) By the combined action of these two mechanisms he entire O_2 stores within the considerable venous blood volume may without eripheral O_2 losses, be mobilized for subsequent delivery to the brain and myo- ardium thus greatly prolonging the time that the animal can stay submerged stimations in some diving species of their total oxygen stores, the oxygen demands of their vitally important tissues and their survival time during prolonged submer- ion strongly suggest that virtually the total O_2 reserve, of which the O_2 content of he venous blood forms an important part, is exhausted before the animal succumbs o anoxia (*cf* Andersen 1966)

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L. R Yonce Ph D was supported by a Special Fellowship No 1 F3 HE-29, 358, from the National Institute of Health Bethesda Md, USA
AB Hassle Göteborg Sweden has generously covered the employment of a technician

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From the Institute of Neurophysiology, University of Copenhagen, Denmark

The Refractory Time in the Visual Cortex of Albino Guinea-Pigs[1]

By

SVEND E SIMONSEN[2]

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Abstract

SIMONSEN, S E *The refractory time in the visual cortex of albino guinea pigs* Acta physiol scand 1969 *75* 387—396

The absolutely refractory time of the summed cortical response, tested with paired supramaximal stimuli to the contralateral optic nerve of chloralose anesthetized guinea pigs, was 86±3 msec The absolutely unresponsive time tested with paired white flashes to the contra lateral eye was 96±6 msec The difference was thought to be due to the fact that the flashes

it could engulf the primary response to the testing flash and make the determination of the unresponsive time impossible

Animal experiments show that the cortex has a lower resolving power in time than the peripheral portions of the visual system and thus determines the critical fusion frequency of the system as a whole The summed response to flashes in anesthetized monkeys could be driven up to about 60/sec in the optic nerve and lateral geniculate body whereas in the cortex the maximum rate followed was 34/sec (Walker *et al* 1943) In the decerebrate cat responses in the retina, optic tract and lateral geniculate responded up to 100/sec, in the cortex not over 40/sec (Lindsley 1953) The ability to follow every shock of a pair or train to the optic nerve decreased at each synaptic station in the visual system (Grusser, Hellner and Grusser-Cornehls 1962), and Brazier (1963) showed 100/sec responses in the optic tract and lateral geniculate but not in the cortex of unanesthetized cats

The frequency at which fusion occurs is much lower in a rod retina such as that of the guinea pig or rat than in a cone retina such as that of the squirrel (Born

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schein and Szegvári 1938 Gouras and Gunkel 1962) or pigeon (Dodt and Wirth 1954) under the same conditions The aim of the study reported here was to see whether the temporal resolving power of the cortex was as slow as that of the eye in an animal with a pure or nearly pure rod retina or whether it was slower To that purpose the refractory time of the summed cortical potential of albino guinea pigs was tested with paired supramaximal shocks to the optic nerve and with paired flashes to the eye

Method

Animals and operative procedures

Successful experiments were carried out on 12 albino guinea pigs weighing 500 to 800 g They were anesthetized with chloralose 1 % and urethane 3 1/2 % in an initial dose of 8 ml/kg supplemented during the operation by a mean of 4 ml/kg The animals were tracheotomized. Flashes were delivered to one eye and electrical stimuli to the other optic nerve and the gross response was recorded from the striate area contralateral to the stimulus The left eye was dripped with 0.5 % atropine sulphate and was covered to allow dark adaptation for about 6 hrs before the experimental run The anterior half of the right eye was cut away the contents gently wiped off with cotton swabs and the sclera was sutured to the adjacent tissue to expose the optic nerve at the cup The tissue was kept moist with a 0.9 % NaCl solution.

The animal's head was fixed by stout pins in each ear and by a screw clamp around the upper incisors. Two circular craniectomy openings were made to expose the area striata on both sides The dura was left intact and was covered with a thin layer of mineral oil The light in the room was then extinguished and further procedures were carried out by weak red light The left eye was uncovered the eye lids removed and the eye was stabilized with sutures at the sclero-corneal edge The animal lay on a heating pad and the rectal temperature was maintained at 37 to 38° C The epidural temperature measured about every hour with a thermistor was found to be at 34 to 34.5° C The light stimulating device was placed in position and a tube from it to a rubber ring around the eye protected the eye from light except from the flash

Light stimulation The light source was a Sylvania glow modulator tube type R 1130B triggered by 50 mA from a square wave generator The flash duration was 10 msec the intervals between flashes of a pair were up to 200 msec and the pairs were delivered every 10 sec The maximum energy of white light measured with a vacuum photomultiplier (Packmann Olsen and Rosenfalck 1957) by comparison with a calibrated standard lamp was 695 µW/cm² The color of the flashes was varied by interposing across the parallel beam three broad interference filters with peak transmission with the light source at 450 510 and 590 mµ passing 26 mµ at half transmission and 50 mµ at 5 % transmission The maximum intensities of green and yellow flashes were of about equal brightness by reference to Weale's (1953) bleaching experiments in albino guinea pig eyes 1.7 µW/cm² for green and 16.1 µW/cm² for yellow [illegible] ements [illegible]

gave too weak intensities as equal in brightness to green and yellow

Six grey filters allowed the intensity of the light to be diminished in steps of one decade [illegible] cornea to illuminate the [illegible] mm [illegible] erve were two steel wires [illegible] ode was inserted through [illegible] as placed on the surface [illegible] lly screened transformer (Gu d 1954 ratio 1 1 5) was used 0.5 msec rectangular pulses were delivered from a stimulator (Disa Multistim) The strength used to determine the refractory period was 3 to 4 times threshold The frequency of stimulation was once in 5 sec

Recording To record the summed cortical response a silver ball electrode suspended on a spring was placed lightly on the dura at the point of best response usually 5 to 6 mm lateral to the sagittal sinus and in the center of the angle between the sagittal and transverse sinuses The indifferent electrode was a steel needle inserted in the back of the neck

" (balanced)
f 10 000 Hz
with short
oscilloscope
(Disa) and photographed on 35 mm film with a Dumont camera. Ten responses to the same pair of stimuli were superimposed The stimuli were signalled with a marking pulse from the generator and a photo cell displayed the flash

Measurement The record ngs were enlarged 6 times and the superimposed responses were traced for measuring

Results

A Electrical stimulation

1 Cortical responses to a single shock to the optic nerve

The summed cortical response to an electric shock to the optic nerve was diphasic, the initial phase being positive (Fig 1) When the stimulus was of threshold intensity a small positive deflection appeared after a latency of about 10 msec (3 8 in Fig 1) A slight increase in stimulus intensity (3 9 in Fig 1) evoked a second positive response smaller than the first and with a latency of about 30 msec As the responses increased the second response was superimposed as a notch on the descending phase of the first positive potential The peak to-peak amplitude of the response increased abruptly with increasing strength of the stimulus to 3 to 4 mV, the latency decreased to 6 msec (Fig 2) and an initial small negative phase appeared Both the latency and the amplitude reached a level when the strength of the stimulus was about two times threshold thus strengths more than two times threshold were supramaximal

2 Cortical responses to the second of paired shocks to the optic nerve

The conditioning stimulus was three to four times threshold *i e* supramaximal

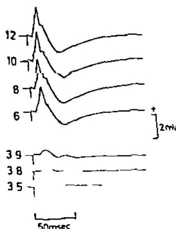


Fig 1 The gross response in striate cortex to stimuli to the contralateral optic nerve The stimulus strengths are given to the left in arbitrary units Each response has been drawn from 10 superimposed sweeps

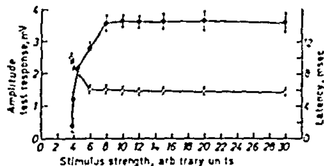


Fig 2 The amplitude (filled circles) and latency (open circles) of the test response to stimuli to the contralateral optic nerve as a function of stimulus strength The conditioning shock was supramaximal The vertical bars indicate the S.E. of the mean

When the testing stimulus was slightly above threshold a response appeared when the interval between stimuli was 180 to 200 msec, the amplitude of the response to the testing stimulus increasing about linearly with the interval between the stimuli (1 4 in Fig 3) With increasing strength of the testing stimulus, the response appeared earlier and the amplitude increased more abruptly (compare 2 1 in Fig 3) When the testing stimulus was three times threshold a response to it appeared when the stimuli were 100 msec apart Extrapolating the linear portion of the increase in amplitude back to the abscissa gave the refractory times shown in B of Fig 3 Further increase in the strength of the testing stimulus did not shorten the refractory time indicating that the absolutely refractory time of the most excitable cells was being measured Such a strength/interval curve was obtained in every instance to ensure that both stimuli was supramaximal The mean absolutely refractory time was 86 ± 3 msec (S E of the mean)

The response to the testing stimulus did not disappear entirely when its amplitude had ceased to diminish linearly with shortening intervals between stimuli It persisted as a tiny response whose amplitude remained constant down to 3 msec the smallest

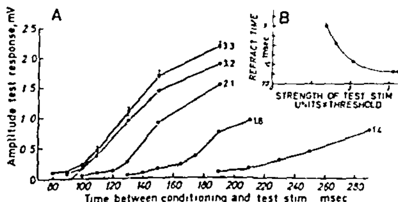


Fig 3 A The amplitude of the test response as a function of the time between paired stimuli to the contralateral optic nerve at different stimulus strengths given beside each curve in units of threshold The conditioning shock was supramaximal B The refractory periods in the same experiment obtained by extrapolating (stippled line) the linear increase in amplitude back to the base line

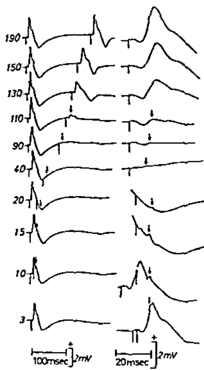


Fig 4 The gross response in striate cortex to paired shocks to the contralateral optic nerve The numbers to the left give the time between paired stimuli in msec The test responses are displayed to the right on a fast sweep Each response has been drawn from 10 superimposed sweeps The small response to the test shock down to intervals of 3 msec is indicated by an arrow

interval tested (Fig 4) This small response was not artefact That its amplitude remained unchanged with shortening intervals rendered unlikely that it was of cortical origin Probably it was a response conducted passively either from the geniculate or from the radiation fibers This possibility was not tested by recording after decortication

B Light stimulation

1 *Cortical response to single white flashes of high intensity*

The summed cortical response to white flashes of the highest intensity available was diphasic with an initial positive phase (Fig 5) a peak to-peak amplitude of 2 to 3 mV and a latency of 20 msec The amplitude decreased and the latency increased linearly with decreasing intensity except between the highest intensity steps when the change was more gradual (Fig 6) The fact that neither the amplitude nor the latency of the response had reached a level with the most intense white flashes suggested that they were not supramaximal

Some 150 to 200 msec after the flash a second response appeared (marked 2 in Fig 5) consisting of a positive potential with one or two peaks whose form duration and amplitude could vary considerably in the same animal and from experiment to experiment I did not find constant relations with these variations except that the

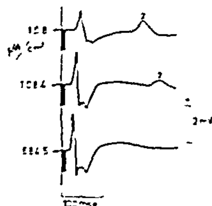


Fig. 5. The gross response in striate cortex to contralateral white flashes of different intensities (left). The second response is marked with 2. Each response has been drawn from 10 superimposed sweeps.

second response seemed to be larger when the light was weak. The second response was not an *off* since an *off* response did not appear unless the flashes lasted 120 msec or more.

2. *Cortical responses to the second of paired white flashes of high intensity*

The responses to the second flash were positive or diphasic and their onset was sometimes poorly defined especially when they were small. The latency had therefore to be measured to the first positive peak, and the amplitude was measured from the base line to the positive peak.

A response to the second flash was just discernible when the flashes were separated by 105 msec on the average. The amplitude increased linearly with increasing inter-flash intervals to a mean of 140 msec, and then more gradually or erratically. Extrapolating the linear portion of the curve of amplitude back to the time line in each case gave the absolutely unresponsive time, a mean of 96 ± 6 msec, or 10 msec longer than the absolutely refractory time tested with paired supramaximal shocks to the optic nerve. However, the white flashes of high intensity were not supramaximal. In the last experiment therefore the output from the glow modulator tube was tripled by increasing the current across it. In that animal the amplitude did not increase and the latency did not decrease further with the further increase in light intensity, suggesting that the white flashes were maximal. The unresponsive time decreased from 100 msec when the flashes had the highest intensity usually available to 90 msec

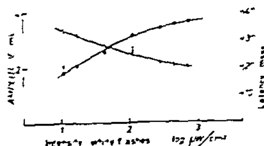


Fig. 6. The amplitude (closed circles) and latency (open circles) of the gross response in striate cortex to 10 msec white flashes to the contralateral eye as a function of the intensity of the flashes. The vertical bars indicate the S.E. of the mean.

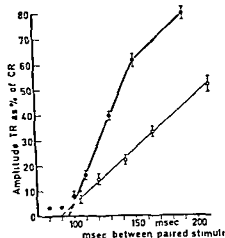


Fig 7 To show the refractory time obtained in the same animal by supramaximal stimuli to the contralateral optic nerve (filled circles) and supramaximal flashes to the contralateral eye (open circles. 10 msec white flashes, intensity 2093 μW/cm²) *Ordinate* amplitude of the test response as per cent of the conditioning response *Abscissa* msec between paired stimuli The vertical bars indicate the S E of the mean.

when the flashes were 50 %, 100 % and 300 % more intense, indicating that the absolutely unresponsive time had been reached, it was then the same whether paired flashes or paired shocks to the optic nerve were used—90 to 95 msec (Fig 7)

3 *Cortical responses to blue, green and yellow paired flashes*

Flashes of three colors (λ max 450 mμ, 510 mμ and 590 mμ) evoked cortical responses of the same size, about 1 5 mV, and with the same latency, about 30 msec. There was no difference between the three colors in the cortical irresponsive time, which was 125±3 msec In one experiment the white flashes were damped to evoke a cortical response with the same amplitude as that evoked by colored flashes The form and latency of the responses were then the same and the irresponsive time was not significantly different

4 *Interaction between the response to the testing flash and the second response to the conditioning flash*

As the amplitude of the response to the testing flash decreased with diminishing time between the flashes, the latency increased to about 40 msec Knowing the range of the latency of the response to the testing flash helped to distinguish it from the second response to the conditioning flash. Sometimes the identification could not be made and these experiments had to be discarded

The two responses—second response to the conditioning flash and response to the testing flash—seemed to affect each other in various ways when they encroached on each other as illustrated in Fig 8 where 2 is the second response to the conditioning flash and a stippled line indicates the response to the testing flash. i) The second response to the conditioning flash could become smaller when it immediately preceded the response to the testing flash (*B* and *C* of *Fig 8*) ii) The two responses seemed to sum when they coincided as at flash intervals of 150 and 140 msec in B and C of Fig 8 iii) The response to the testing flash could become considerably smaller, as in A of Fig 8, as it was drawn closer to a prominent second response to the conditioning flash and it could be engulfed by that response and not be found with any certainty when it should have reappeared before the late response to the conditioning flash (it may be small at intervals 130 and 120 msec in A of Fig 8) iv) A

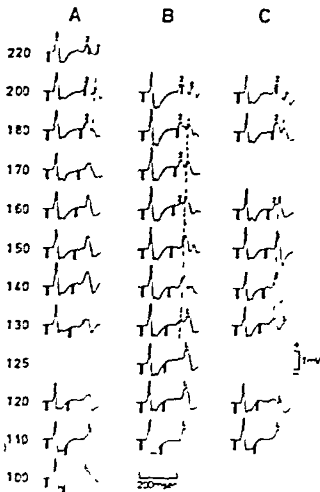


Fig. 8. To show various types of interaction between the test response (marked with a stippled line) and the second response to the conditioning flash (marked with 2). Gross responses in the striate cortex in the same animal to paired flashes to the contralateral eye. A. Blue flashes, 2 µW/cm². B. Blue flashes 54 µW/cm². C. White flashes 108 µW/cm². The flashes are indicated by the black vertical bars. The times in msec between paired flashes are written to the left. The late response is indicated by L.

prominent and puzzling interaction between the two responses was the appearance and marked increase in size of a late response (marked L in Fig. 8) which came too early to be a second response to the testing flash and which was too late to be a second response to the conditioning flash unless it had been considerably delayed. This late response was largest at small intervals when the response to the testing flash had disappeared. Possibly the second responses to both the conditioning and testing flashes appeared after a latency intermediate between the normal latencies and then summed to give the large late response.

Discussion

The recovery time of the summed cortical response: is it determined by the eye or the brain?

Pearlman (1963) found an absolutely refractory time of about 70 msec in the cortex of unanesthetized rabbits tested with paired supramaximal shocks to the optic nerve; the recovery time was of the same order when tested with paired flashes, though the time between flashes was not varied in small steps. The absolutely refractory time of the summed cortical response in my anesthetized guinea pigs, tested with paired

supramaximal shocks to the optic nerve, was 86±3 msec, 10 msec less than the unresponsive time tested by white flashes But these flashes were not of maximum intensity since neither the amplitude nor the latency of the response had reached a level In the final experiment the absolutely unresponsive time was measured since it did not change when the intensity of the flashes was increased from twice to three times the usual intensity The refractory period of the summed cortical response was then the same whether shocks to the optic nerve or white flashes were used Therefore the recovery time of the summed cortical response reflects the temporal resolving power of the central pathways

Locus of the slow cortical recovery time

In cats (Lindsley 1953, Brazier 1963) and monkeys (Walker *et al* 1943) the summed response follows higher flicker frequencies in the eye or optic tract and in the geniculate than in the cortex, the fusion frequency being about twice as high in the geniculate as in the cortex The conclusion seems reasonable that the long recovery time of the summed cortical potential of albino guinea pigs is a characteristic of the cortex and not of more peripheral stations in the visual pathway

Paired flashes and trains of flashes

Though paired stimuli can test the temporal resolving power of nervous tissue, the results differ quantitatively from findings with trains of stimuli Bornschein and Szegvari (1958) found an unresponsive time of 40 msec for the ERG evoked by paired flashes in the guinea pig whereas the fusion frequency tested with flickering light of the same intensity corresponded to an irresponsive time of 14 to 20 msec The difference corresponds well with findings in humans (quoted in Brown 1965 p 264)

The effect of anesthesia Anesthesia is certainly an important variable Gastaut *et al* (1951) found a cortical unresponsive time of 20 msec in unanesthetized cats, of 100 msec in choralose and dial anesthetized cats These authors show the difference in the cortical excitability cycle under the two anesthetics Since I studied the recovery time with paired flashes and with paired shocks in the same animal and with the same anesthetic, the main result that the recovery time was the same is probably valid

Recovery time after colored flashes Blue green and yellow flashes which evoked cortical responses of equal amplitude gave identical recovery times Paired colored flashes were used to provide a basis for comparison with ground squirrels, animals with a cone retina However the light source turned out to be too weak to be usable in these cone animals and this aspect of the problem was studied subsequently with a different light source (Hammerberg in preparation)

The secondary response A delayed second response to flash has been described repeatedly (reviewed in part in Torres and Warner 1962), it resembles the secondary response to sciatic stimulation which has a much longer unresponsive time than the primary response (Forbes and Morison 1939) The second response seen in anesthesia

was called Type I by Torres and Warner (1962), who found it associated with the primary response both in the cortex and in subcortical structures including the optic tract The late response has indeed been described in single retinal ganglion cells as a secondary discharge after the primary discharge and an "Entladungspause", summation of this secondary discharge with the primary discharge of the subsequent flash has been described (Grüsser and Rabelo 1958), as I saw it I made no systematic study of these interactions and can add little to what is known about the secondary response It did however, exist in guinea-pigs, it made the determination of unresponsive time difficult and could make it impossible, for when the primary response to the testing flash disappeared in the secondary response to the conditioning flash and could not again be identified at shorter intervals between flashes, the irresponsive time could not be determined

I wish to thank Dr Lennox Buchthal for help and guidance during the progress of this work and in the preparation of the manuscript My thanks are due to Dr A M Rosenfalck for substantial help in designing and assembling the equipment and to Dr Buchmann for measuring the energy and spectral composition of the flashes

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Relationship between Blood Pressure and Diameter of Ascending Aorta in Normal and Hypertensive Rabbits

By

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Abstract

AARS, H *Relationship between blood pressure and diameter of ascending aorta in normal and hypertensive rabbits* Acta physiol scand 1969 75 397—405

Earlier studies of strips from rabbit aorta suggest that hypertensive resetting of aortic baro- [illegible]

remained linear to near hypertensive control pressures This indicates that at pressures below approximately 130 mm Hg, stretching of aortic wall elements—and presumably stimulus to [illegible]

amplitude and this relationship did not differ significantly in normal and hypertensive rabbits.

Examination of strips from the aortic arch baroreceptor area has shown that in hypertensive rabbits with reset aortic nerve activity, aorta is stiffer and thicker than normal (Aars 1968b) It was therefore suggested that the resetting was due to hypertensive changes in the receptor area

In order to check whether the aortic pressure/diameter relationship in hypertensive rabbits is also altered in physiological conditions, in the present work aortic blood pressure and diameter of ascending aorta have been examined with the circulation intact by means of an ultrasonic diameter measuring gauge

Material and methods

Experiments were performed in 11 normotensive and five hypertensive adult rabbits with mean body weights of 3760 g and 4000 g respectively The rabbits were made hypertensive by wrapping one kidney in silk and removing the other about four weeks later (Aars 1968a) For recording of aortic diameter, two piezo-electric crystals with resonant frequency of 2 Mc/sec

were glued to ascending aorta as near the arch as possible with a thin layer of methyl-2-cyanoacrylate (Eastman 910 Adhesive) (Aars and Leraand 1968). The transit time of ultrasonic pulses between the crystals was measured with a sensitivity of 1 mm deflection on the recorder for each 0.02 mm change in aortic diameter. The frequency response was about 40 cps. Calibration was based on a 1500 m/sec velocity of the pulses. In six normal and three hypertensive rabbits anesthetized with pentobarbitone (Nembutal) 30 mg/kg i.v. and ether the crystals were implanted about one week before examination of the pressure/diameter relationship. In five normal and two hypertensive animals the crystals were implanted on the day the pressure/diameter relationship was examined. The animals were anesthetized with a mixture of 1% chloralose and 25% urethane 3 ml of each per kg. When necessary urethane was supplemented. The chest was then closed and the respirator disconnected before examination of pressure and diameter. Chloralose and urethane were also used for the animals in which the diameter measuring gauge had been implanted one week earlier.

Aortic blood pressure was measured with a Statham transducer via a catheter introduced into the right common carotid artery. Catheters of standard length and calibre were used. Before insertion the catheters were damped by tightening a screw clamp until abrupt release of 100–125 mm Hg suction produced a 5—10 mm Hg overshoot without significantly reducing the maximal dP/dt. This procedure was used in all but one normal and two hypertensive rabbits in which the catheters were undamped. The first stable pressure level recorded was used as control pressure for each animal. During the experiment pressures were altered by stepwise removal and reinfusion of blood through a cannula in the right jugular vein. Recordings were made after more than 30 sec at each pressure level and lowest diastolic pressure, pulse pressure (ΔP), smallest diastolic diameter (D), pulsatile diameter increase (ΔD) and heart rate were measured on the curves. In 10 normal and three hypertensive animals the first derivatives of blood pressure (dP/dt) and diameter (dD/dt) were also recorded. A 4-channel Sanborn recording machine was used at a paper speed of 25 or 50 mm/sec.

At the end of each experiment the heart was removed and divided into three parts: the atria, left ventricle with septum and right ventricle. The parts were dried with filter paper and weighed. A 3–4 cm length of descending aorta was excised, cleared of loose connective tissue and opened. The weight of the tissue was determined to the nearest 0.01 mg after rinsing with 0.9% saline and drying with filter paper.

The aortic tissue was stored in a vacuum extractor and the weighing was repeated three months later. Aortic tissue reactions to the adhesive were investigated by microscopy.

Results

Pressure/diameter relations were examined in the hypertensive animals 8—11 days after nephrectomy. At this time blood pressures ranged from 200/150 to 150/125 mm Hg with a mean value of 170/138 mm Hg for the five animals. Mean blood pressures in the group of normotensive animals were 119/95 mm Hg (range 132/105—105/87 mm Hg).

Recordings of aortic blood pressure and diameter in a normotensive rabbit are shown in Fig. 1. It can be seen from the figure that with each systolic pressure rise aortic diameter increased about 0.6 % above lowest diastolic diameter at control pressure. At a lower pressure the pulsatile variations were increased to about 2.8 % of diastolic diameter. The maximal rate of change of diameter (dD/dt) had also increased but maximal rate of change of blood pressure (dP/dt) remained practically unchanged.

Pressure/diameter curves for all normotensive and hypertensive animals are presented in Fig. 2. The curves were linear below inflexion points with correlation coefficients ranging from 0.95 to 0.99. The slope of the linear parts of the curves varied considerably in individual rabbits and neither slope nor per cent increase in diameter in response to a given elevation of pressure showed significant differences between normal and hypertensive rabbits. Inflexion points (here defined as the point where a 15 mm Hg rise of diastolic pressure led to a 0.2 mm increase in diastolic

Rate of change per sec
dD/dt
8
0
−8
dP/dt
2000
0
−2000

Aortic diameter (D) mm
93
89
85

Aortic blood pressure (P) mm Hg
100
0

1 sec

Fig 1 Original recording of aortic blood pressures ascending aortic diameter and rate of change of diameter (dD/dt) and pressure (dP/dt) in a normal rabbit before (left panel) and after moderate bleeding (right panel) In both cases, dD/dt is recorded together with mean blood pressure

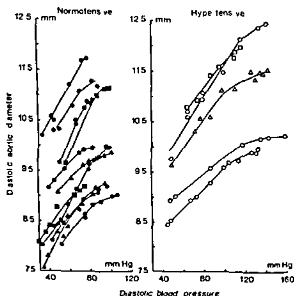


Fig 2 Relationship between diastolic blood pressure and diameter in ascending aorta in 11 normal and five hypertensive rabbits. Lines drawn to best visual fit

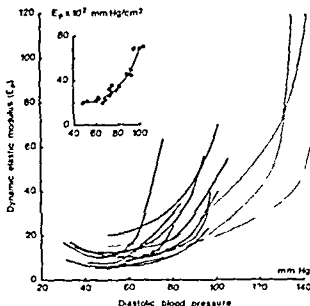


Fig 3 Dynamic elastic modulus $\left(E_p = \frac{\Delta P \times D}{\Delta D}\right)$ at various diastolic blood pressures in 10 normal rabbits (heavy lines) and three hypertensive rabbits (dotted lines) Lines drawn to best visual fit of individual observations as illustrated inset

diameter) were reached at higher pressures in hypertensive animals (108—135 mm Hg) than in normal animals (70—100 mm Hg) No consistent differences were found between animals studied one week after or on the day the diameter gauge was implanted

Pulse pressures were similar at control pressures in the two groups (Table I) but tended to fall to lower values in the hypertensive than in the normal rabbits during bleeding Aortic diameter pulsations were greatest at low pressures and reached a mean of 2.2 % of diastolic diameter at 55 mm Hg in the normal group In all animals the pulsations diminished with increasing diastolic pressure Pulsations at control pressures averaged 0.8 % in normal animals and 0.5 % in hypertensives There were great individual variations and pulsations were not significantly different in the two groups

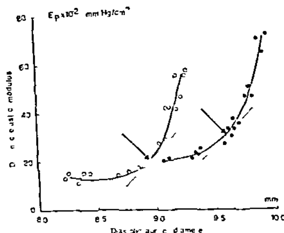
Pulse pressures and diameter pulsations were combined in calculation of the dynamic elastic modulus E_p[1] Peterson Jensen and Parnell 1960) E_p ranged from 500 to 2000 mm Hg/cm² at low pressures with about equal values in normal and hypertensive animals (Fig 3) It increased with increasing blood pressures and at control pressures was higher in hypertensive than in normal rabbits

Dynamic elastic modulus was also related to diastolic diameter This procedure gave less variation in individual rabbits and made it easier to determine the position of the inflexion point of the E_p curves This was effected by finding the point where a 0.1 mm rise in diastolic diameter produced a rise in E_p of 5000 mm Hg/cm² as illustrated for two animals in Fig 4 The diastolic blood pressures corresponding to

[1] Dynamic elastic modulus

$$E_p = \frac{\Delta P \times D}{\Delta D} \text{ mm Hg/cm}^2$$

ΔD is the increase above diastolic diameter D produced by the pulse pressure ΔP



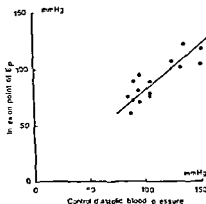


Fig 4 Fig 5

Fig 4 Dynamic elastic modulus (E_1) related to diastolic aortic diameter in two normal rabbits Inflexion points (arrows) are determined as the points on the curves where a 0 1 mm rise of diastolic diameter produced a 500 mm Hg/cm² rise in E_1

Fig 5 Relationship between control blood pressure and inflexion point in 10 normal (solid symbols) and five hypertensive rabbits (open symbols) Lines drawn to best visual fit

the diameters thus found were determined from the pressure/diameter curves in Fig 2 Mean inflexion points of E_1 were found at 78 and 109 mm Hg in normal and hypertensive rabbits respectively E_1 failed to reach inflexion point in one normal animal because of low blood pressures during the experiment As seen in Fig 5 the shift of inflexion points corresponded well to the elevation of arterial pressure the relationship between control diastolic blood pressures and inflexion point pressures remained constant.

The maximal rates of change of pulse pressure and diameter were recorded in 11 normal and three hypertensive rabbits (Table I) Both parameters were found to reflect the differences in the amplitude of pulse pressure (Table I) and diameter respectively in the two groups of animals Calculation of the relationship between amplitude and maximal speed of the diameter changes $\left(\Delta D \big/ \frac{dD}{dt}\right)$ at various blood pressures showed large variations between individual rabbits In most animals the relationship was constant at all pressures In others the ratio increased with increasing diastolic pressure However the $\Delta D \big/ \frac{dD}{dt}$ showed no consistent differences between normal and hypertensive animals either in mean values (0 040 and 0 039 respectively) or in its relationship to blood pressure

Wet and dry weight of aortic tissue was obtained in eight normal and three hypertensive rabbits Dry weight constituted a significantly ($P<0\ 01$) smaller per cent of wet weight in the group of normal rabbits (24 %) than in the hypertensive rabbits (29 %)

TABLE I Variations with diastolic blood pressure (BP) in pulse pressure (ΔP) and maximal rates of change of pressure (dP/dt) and aortic diameter (dD/dt) during stepwise bleeding and reinfusion. Mean of 11 normal (N) and three hypertensive (H) rabbits. Control diastolic pressures in the two groups were 87—105 mm Hg and 125—150 mm Hg respectively

BP mm Hg	ΔP mm Hg		dP/dt mm Hg/sec		dD/dt mm/sec	
	N	H	N	H	N	H
30— 39	23		1581		4.4	
40— 49	25		1646		4.9	
50— 59	23	16	1579	1100	5.5	3.5
60— 69	25	22	1678	1431	3.5	3.9
70— 79	24	18	1642	1075	3.0	4.2
80— 89	27	20	1732	1221	3.0	3.3
90— 99	30	20	1803	1279	2.3	2.5
100—109	37		1970		2.4	
110—119		21		1409		1.8
120—129		30		1438		1.1
130—139		29		1686		1.4
140—149		34		2048		1.6

Left/right ventricle weight ratios were higher in hypertensive (mean 4.2, SE 0.17) than in normal rabbits (mean 3.2, SE 0.1).

After a few hours application of Eastman 910 Adhesive, histological examination showed no ill effects on the aortic wall. After one week, elastic tissue (stained with Drury and Wallington's (1967) modification of Gomori's aldehyde fuchsin stain) and collagenous tissue (van Gieson's stain) were still unaffected, but there was a sharply localized necrosis of smooth muscle cells evident in sections stained with H&E and Mallory's PTAH.

Discussion

The shift of inflexion points of the pressure diameter curves was the most striking finding in the present study. The inflexion point is believed to coincide with the pressure where the elastic tissue is fully unstretched, and where further distension of the artery is limited to an increasing extent by the less distensible collagenous tissue (Moret, Cuenod and Duchosal 1957, Roach and Burton 1957, Moret 1964, Wolinsky and Glagov 1964). It may consequently be assumed that the stretching of aortic tissue at this point was nearly equal in all animals, more so than at any other determinable point. At identical pressures below hypertensive inflexion points, the aortic tissue (and presumably the baroreceptors) was accordingly less stretched in hypertensive than in normal animals. A similar conclusion was arrived at in the load length studies of aortic strips (Aars 1968b). Strips from hypertensive rabbits required a

TABLE II Observations of dynamic elastic modulus (E_P) and pulsatile diameter variations in per cent of diastolic diameter in intact thoracic aorta Values of E_P to nearest 100

Author(s)	Method of measurement	E_P mm Hg/cm²	Blood pressure mm Hg	Pulsatile diameter variations %	Location, species
1 Timm (1947)	X ray		not stated	14	aortic arch, cats and dogs
2 Rushmer (1955)	mercury in rubber strain gauge		100 (diastolic)	4*	descending aorta, dog
3 McDonald (1960)	X ray		not stated	5—7	thoracic aorta, dogs
4 Peterson, Jensen and Parnell (1960)	transformer coil	1200	not stated	not stated	descending aorta, dog
5 Barnett, Mallos and Shapiro (1961)	electrical strain gauge caliper		not stated	2 5—4	descending aorta, dogs
6 Greenfield and Patel (1962)	as No 5	700*	95* (mean)	5 5*	ascending aorta, humans at operation
7 Luchsinger, Sachs and Patel (1962)	X ray	700*	99* (mean)	2 55	descending aorta, humans
8 Mallos (1962)	as No 5	400*	100 (diastolic)	7 5*	thoracic aorta, dog
9 Ohlsson (1962)	X ray		not stated	14	ascending aorta, dogs
10 Patel *et al* (1963)	as No 5	600	97* (mean)	4 7*	ascending aorta, dogs
		800**	95** (mean)	3 5**	descending aorta, dogs
11 Remington (1967)	mutual inductance coils on steel blades	700*	40 (diastolic)	7*	descending aorta, cat
12 Aars	ultrasonic gauge	2000—6000	95 (diastolic)	0 8	ascending aorta, rabbits

* Figures calculated from published data

** Calculated from their observations in upper, middle and lower part of descending thoracic aorta

higher load to reach the same stretching of aortic wall elements as strips from normal animals However shift of inflexion points of the load length curves was not seen This discrepancy between the two investigations might reflect the difference between the static and dynamic experimental conditions but could also result from higher control blood pressures (average 196/153 mm Hg) in the previous group of hypertensive rabbits Left ventricular hypertrophy was also more pronounced in that group

The higher than normal dynamic elastic moduli (E_t, Fig. 3) at and above control pressures in the hypertensive animals and the individual variations of E_t in this *pressure range are of relevance to the baroreceptors. A high E_t means smaller diameter pulsations* and less stimulation of the receptors in response to a given pulse pressure. Below control pressures pulse pressures were higher in normal than in hypertensive animals. A high pulse pressure will stretch the aortic wall further into a less distensible range than will a low pulse pressure. E_t is therefore not directly comparable in the two groups at low blood pressures.

Pulsatile diameter variations were smaller in the rabbit's ascending aorta than those usually found in humans, cats and dogs (Table II). Similarly E_t was up to 10 times as high (Table II). Patel *et al.* (1963) examined aorta in 6 dogs and found E_t to be on average 800 g/cm², or about 600 mm Hg cm², and similar figures can be calculated from single observations made by others. Results obtained in other series of rabbits are not available for comparison.

Baroreceptor activity is related to both diameter and rate of change of diameter in the vessel (Peterson 1960). It would therefore be of interest to know if the relationship *between amplitude and maximal rate of change of the pulsatile diameter variations* was different in normal and hypertensive rabbits, especially because the increased aortic water content might be expected to influence this relationship (Peterson 1963). No significant changes were found. The relationship between aortic nerve activity and aortic diameter may thus be compared in normal and hypertensive rabbits without reference to the actual rate of change of diameter.

Tissue reactions to methyl 2-cyanoacrylate (Eastman 910 Adhesive) have been widely tested. Early reports showed few ill effects of using the adhesive in the repair of arterial incisions and as an adjunct to suture in arterial anastomoses (Nathan *et al.* 1960, Healey *et al.* 1962, Matsumoto *et al.* 1963), but later reports have stressed the occurrence of localized necrosis of the vessel wall (Reddy and Ecker 1966, Lehman and Hayes 1967). The adverse reactions to some extent depend upon the quantity used (Braunwald 1966, Goetz, Weissberg and Hoppenstein 1966). In the present study the adhesive was topically applied in very small amounts, and the larger part *of aortic circumference was left free. Elastic and collagenous tissue, the two elements of greatest importance to the aortic pressure diameter relationship, was unaffected* by the adhesive. The localized damage to smooth muscle cells had not interfered with aortic wall dynamics, as no differences in pressure diameter relationship were observed between animals with the crystals implanted the same day or one week before. The main assets of the present method are that the transducers can be implanted, and that it gives precise diameter signals which are easy to calibrate and are easily read. The crystals may be left in place for a long time, and may therefore also be used for measurements of aortic diameter in awake animals. The results obtained with this method showed significant differences in pressure/diameter relationship in ascending aorta of normal and hypertensive rabbits, differences which presumably have great consequences for aortic baroreceptor activity in hypertension.

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Relationship between Aortic Diameter and Aortic Baroreceptor Activity in Normal and Hypertensive Rabbits

By

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Abstract

AARS, H. *Relationship between aortic diameter and aortic baroreceptor activity in normal and hypertensive rabbits.* Acta physiol. scand. 1969. 75. 406—414.

Studies of aortic nerve activity (Aars 1968a) and the load/length relationship of strips from the aortic wall (Aars 1968b) have suggested that the hypertensive resetting of baroreceptor activity is due to changes in the aortic wall. In nine normal and four hypertensive rabbits, diameter of the ascending aorta, measured with an ultrasonic gauge, was therefore recorded together with aortic baroreceptor activity. Aortic nerve activity was closely related to aortic diameter in individual rabbits, but wide variations in pressure/diameter relationships made it difficult to compare diameter and activity in a group of rabbits. Assuming that stretching of the receptor area would be almost equal in all rabbits at the inflexion point of their pressure/diameter curves, the diameter corresponding to inflexion point of the curve for dynamic elastic modulus was used as reference diameter. Plotted against per cent of this diameter, the nerve activity showed acceptable uniformity in the group of normal rabbits, and observations in hypertensive rabbits showed less deviation from the normal. The uniformity was further improved by allowing for variations in diameter pulsations. It was therefore concluded that the hypertensive resetting of aortic baroreceptor activity was predominantly due to altered distensibility of the aortic wall.

Recent quantitative studies of aortic baroreceptor activity (Aars 1968a) and examination of aortic strips from the receptor area (Aars 1968b) have suggested that resetting of the baroreceptor activity during hypertension is due to the aortic wall being thicker and stiffer than in normal rabbits. This view has later been supported by demonstration of changed pressure/diameter relationship of intact aorta in hypertensive rabbits (Aars 1968c). If this conclusion is correct, the relationship between aortic baroreceptor activity and stretching of the aortic wall should be equal in normal and hypertensive rabbits. Peterson (1960, 1966) recorded carotid sinus diameter and carotid baroreceptor activity in dogs, but was unable to study these parameters in the same animal before and after establishment of hypertension. Aortic baroreceptor activity has not previously been correlated to diameter in the receptor area.

To test the above-mentioned hypothesis, studies were made of aortic baroreceptor activity and aortic diameter in normal rabbits and in rabbits with renal hypertension

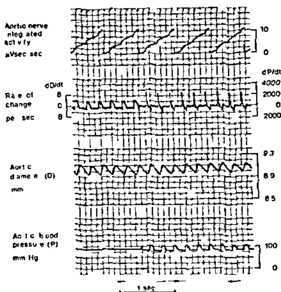


Fig 1 Strip of simultaneous recording of integrated aortic nerve activity rates of change of diameter (left) and blood pressure (right) ascending aortic diameter, and mean and pulsatile aortic blood pressure, in a normal rabbit

The obstacle of having each animal as its own control was overcome by quantitative analysis of whole nerve activity For practical reasons, the diameter was recorded in ascending aorta rather than in the aortic arch

Material and methods

Left aortic nerve activity aortic diameter and aortic blood pressure were simultaneously recorded in nine normal and four hypertensive rabbits Hypertension was produced by wrapping one kidney in silk and removing the other about four weeks later (Aars 1968a) Activity in the whole aortic nerve was recorded with a differential amplifier A quantitative measurement was obtained by rectification and integration of the signals (Aars and Leraand 1968) The transit time of ultrasonic pulses between two crystals glued to aorta was used to

reinfusion of blood through a cannula in the right jugular vein Recordings were made after more than 30 sec at each pressure level Measured mean nerve activity was normalized by calculation to a mean nervous activity per sec at a heart rate of 300 beats per min (Aars 1968a) The first derivatives of blood pressure (dP/dt) and diameter (dD/dt) were recorded together with the other parameters on a 4-channel Sanborn recording machine at a paper speed of 25 or 50 mm/sec

Results

The hypertensive animals were examined 8—11 days after nephrectomy at a time when blood pressure in this group was on average 163/136 mm Hg (range 175/150—150/125 mm Hg) Blood pressure in the control group averaged 122/96 mm Hg (range 132/105—105/87 mm Hg) Heart rates during observations of aortic diameter

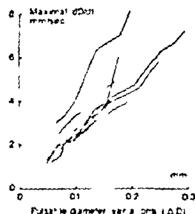


Fig. 4. Relationship between amplitude and maximal rate of change of pulsatile aortic diameter variations in eight normal (heavy lines) and three hypertensive (dotted lines) rabbits. The lines are drawn through mean values of 3–4 observations at each recording level.

0.2 mm increase in diastolic diameter, and the latter inflexion point was found as the diameter (D_L) where a 0.1 mm increase of diastolic diameter led to a rise in E_p of 500 mm Hg/cm² (Aars 1968c). All other diameters were then expressed in per cent of the reference diameter. The last method produced greatest uniformity in the relationship between aortic diameter and aortic nerve activity in normal rabbits, and it was therefore applied also to the hypertensive rabbits. The reference diameter D_L was found at higher pressures in hypertensive rabbits (average 107 mm Hg) than in normal rabbits (average 78 mm Hg), and as shown in Fig. 3B the discrepancy previously observed between the two groups of animals (Fig. 3A) largely disappeared when receptor activity was compared to diameter in this way. Activity started to rise in approximately the same range of aortic diameter, but the lines were not as steep for hypertensive as for normal animals, and the hypertensive rabbits showed less activity than most of the normal rabbits.

Baroreceptors are sensitive to rate of change in diameter, not only to mean stretch. It would therefore be desirable to relate the activity to dD/dt at varying aortic diameters. However, as the recording of dD/dt was not performed in all animals and measurement of dD/dt was less precise than measurement of diameter (Fig. 1), the feasibility of using amplitude rather than rate of change of diameter variations was investigated. In Fig. 4, maximal rate of change has been related to amplitude of diameter variations in eight normal and four hypertensive rabbits. It is evident that in most animals the parameters were linearly related, showing no differences in the two groups. Amplitude of diameter variations, expressed in per cent of diastolic diameter, was therefore applied to the further analysis of receptor activity.

Pulsatile diameter variations were not significantly different at comparable diastolic pressures, but tended to be lower in hypertensive than in normal animals when related to D_L. This is apparent from Fig. 5, where per cent pulsatile variations are plotted against per cent diastolic aortic diameter. Pulsatile diameter variations increased in all animals with reduction of diastolic diameter, but the increase was greatest in normal animals. In other words, pulsatile stretching of the receptor area

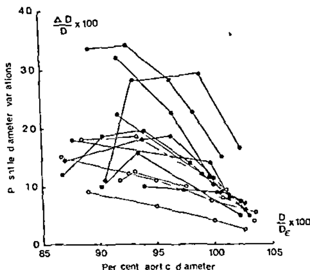


Fig 5 Pulsatile aortic diameter variations at various diastolic diameters in nine normal (heavy lines) and four hypertensive (dotted lines) rabbits Lines drawn as in Fig 4

at comparable aortic diameters was less in hypertensive than in most normal animals This might explain some of the remaining discrepancies between activity in normal and hypertensive animals in Fig 3B Attempts were therefore made to correlate mean nerve activity to the pulsatile diameter variations (100 $\Delta D/D$) at various distensions (100 D/D_E) of the aortic wall

For simplicity the results are illustrated in Fig 6 only at 94 %, 97 %, 100 % and 102 % of reference diameter D_E Observations from hypertensive animals showed no consistent deviations from the common regression lines drawn in Fig 6, and except for 102 % regression equations were not markedly different, whether calculated from normal animals alone or with observations from both groups In particular observations from hypertensive rabbits were now equally scattered around the common mean and not found in the lower part of the normal range as in Fig 3B The correlation between pulsatile diameter variation and mean nerve activity declined with the rise of diastolic diameter and was significant ($P < 0.05$) only at 94 % 97 % and 100 % of D_E Nonetheless it is apparent from the plots in Fig 6 that most of the difference between normal and hypertensive rabbits disappeared when pulsatile diameter variations were taken into account

Discussion

There are two main possible solutions to the problem of baroreceptor resetting in arterial hypertension changes in the receptors and changes in the wall of the receptor area If the aortic wall was completely unchanged in hypertensive rabbits the aorta—and the receptors—should be equally stretched at high blood pressures in hypertensive and in normal rabbits In that case resetting would be at least partly

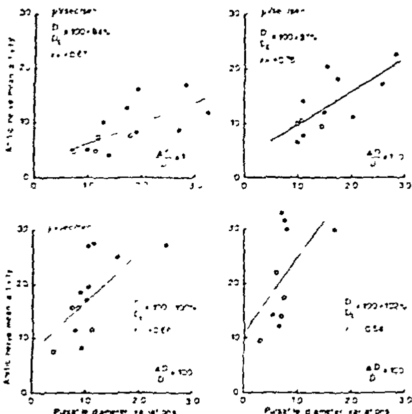


Fig. 6. Mean aortic nerve activity related to pulsatile aortic diameter variations (in per cent of diastolic diameter) at 94%, 97%, 100% and 102% of aortic diameter (100 D/Di, see Fig. 3) in nine normal rabbits (solid symbols) and four hypertensive rabbits (open symbols). r is the correlation coefficient of the regression line calculated for the total population at each diastolic diameter.

due to some kind of adaptation of the receptors. However, as shown in a previous paper (Aars 1968c), the aortic wall *had* changed. Only one week after establishment of hypertension the stretching of aortic wall elements was less than normal at subhypertensive blood pressures and almost equal in normal and hypertensive rabbits at their respective control pressures. Although not proving a causal relationship this is an indication that resetting of receptor activity in these rabbits was due to changes in the wall—*not* in the receptors. In the present paper attempts were therefore made at a more complete correlation of baroreceptor activity to aortic diameter.

Aortic nerve activity was closely related to diastolic aortic diameter in all individual rabbits, but difficulties arose when trying to compare activity and diameter in a group of animals. Assuming that the receptor response to stretch was equal in normal and hypertensive rabbits, a comparison of this kind would ideally require a point of reference that *produced the same degree of receptor stretch in all rabbits*. The inflexion point of the distensibility curves provided obvious possibilities, as this point is believed to coincide with the pressure and diameter at which the elements of

the aortic wall would be approximately equally stretched (Aars 1968c) Attempts were therefore made to use as reference the inflexion point of the diastolic pressure/diameter curves and as the baroreceptors are also sensitive to diameter pulsations the inflexion point (D_I) of the curves for dynamic elastic modulus E_1, plotted against diastolic diameter Both methods gave a reasonable conformity of receptor activity at a given relative diameter in normal rabbits and the second method based on E_p was the best The discrepancy between the two methods might be due to difficulties in obtaining accurate determinations of inflexion points of the diastolic pressure/diameter curves in the present study When related to diameters in per cent of reference diameter D_E most of the difference between aortic nerve activity in normal and hypertensive rabbits disappeared (Fig 3B)

Baroreceptor activity is related to rate of change of pressure (Gero and Gerova 1962), and accordingly also to rate of change of diameter Variations in maximal speed of a given amplitude of diameter pulsation in these animals were small, however and there were no differences between normal and hypertensive rabbits Hence activity was related to amplitude which could be measured with greater precision When variations in diameter pulsations had been allowed for the correlation between diameter and activity was surprisingly good (Fig 6) The poor correlation at 102 % might be due to steeper slope of the diameter/activity curve in this range (Fig 3B) Minor errors in estimation of inflexion point would have greater consequences at this diameter

The regression lines in Fig 6 are not presented as proof that receptor activity is linearly related to diameter variations They are however convenient for comparing the results at various diastolic aortic diameters Diameter was found to be a linear function of pressure below inflexion point D_I (Aars 1968c) With mean or diastolic diameter kept constant activity in this range will accordingly be linearly related to rate of change or amplitude of diameter changes This is consistent with observations made by Gero and Gerova (1962) and with equations for the relationship between blood pressure and receptor activity (Warner 1958 Peterson 1962 Scher and Young 1963) Linearity would be expected to decrease near to and above D_I as was indeed found in Fig 6 Correlation coefficients decreased gradually from low to high percentages of D_I However aortic nerve activity in a group of animals is influenced by many other factors such as variations in the *number of fibres and recording conditions* (Aars 1968a) and complete quantitative equality in all animals is not to be expected Variations in heart rate which might be of importance were large but showed no significant difference between the two groups It is therefore concluded that the resetting of activity in hypertensive rabbits is predominantly due to altered distensibility of aorta in the receptor area

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Acta physiol. scand 1969. 75. 415—426

From the Institute of Pharmacology, University of Uppsala, Sweden

Adrenergic Neuron Blockers and Transmitter Release after Sympathetic Denervation Studied in the Conscious Rat

By

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Abstract

LUNDBERG, D *Adrenergic neuron blockers and transmitter release after sympathetic denervation studied in the conscious rat* Acta physiol scand 1969 75 415—426

13 to 15 hrs after excision of the superior cervical ganglion the postdenervation ptosis in the [illegible] appearance and magnitude There is no correlation between the neurone blocking action of the drugs and their 'delaying effect

Recently the transient and spontaneous adrenergic effects in sympathetically innervated organs following division of the distal neuron (denervation) have been reported and discussed by several authors Linner and Prijot (1955) observed ocular hypotony to occur 24 hrs after high cervical ganglionectomy in rabbits This "ganglionectomy effect", as it has been called has since been more extensively studied and shown to be related to release of stored transmitter from the degenerating nerve terminals (Sears and Barany 1960, Barany 1962) Unprovoked transient secretion (degeneration secretion) from the sympathetically denervated salivary glands in the cat was reported and discussed in similar terms by Coats and Emmelin (1962) The spontaneous transient contraction of the denervated nictitating membrane in the conscious cat has been described and analysed in detail by Langer (1966) Based on pharmacological evidence he too proposed that this phenomenon was due to leakage of transmitter from the terminals and he called it 'degeneration contraction' An analogous phenomenon in the parasympathetic

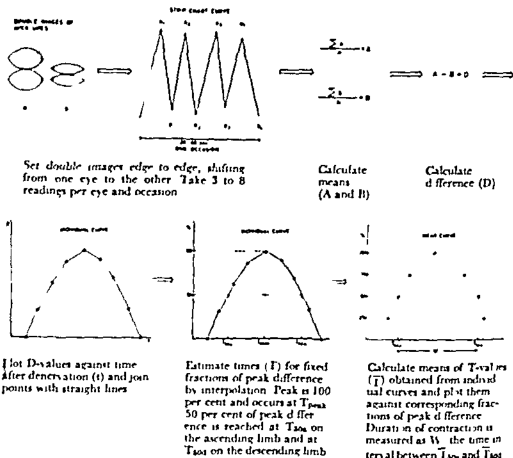


Fig 4 Design of calculation and graphical representation of the degeneration contraction

Calculations

The design of the calculations and the graphical representation of the phenomenon are shown in Fig 4

The degeneration contraction was in most cases studied by analysing the mean curves of groups of 5 animals These were analysed from the following points of view

1 *The time course* Attention was especially paid to the times corresponding to half maximal effect on the ascending part (T_{50a}) and on the descending part (T_{50d}) of the curve

2 *The width* This was measured as the difference between T_{50d} and T_{50a}

3 *The height* This is the maximal value measured on the denervated side or the peak difference between the apertures

4 *The slopes of the limbs* The difference between T_{75a} and T_{50a} and T_{50d} and T_{75d} estimated on the individual curves and averaged, is an expression for the slopes of the ascending and descending limbs respectively of the mean curve The T_{75} values are not on the most linear parts of the curve but they are considered to be more useful than the T_{25} values

The estimation of the sympathomimetic effect

Preliminary experiments showed that this effect usually reached its maximum value about 1 hr after the s.c injection It had comparatively short duration The individual curves were constructed by plotting the mean values of the aperture on the decentralized side from every estimation occasion against the time Because of the inconvenient time of the night no values

were obtained before the injection and the first value was taken after 1 hr, subsequent readings were taken at hourly intervals. The magnitude of the effect was estimated by measuring the difference between the maximal value noted and the average of at least 5 consecutive values taken from the lowest plateau part of the individual curves Mean curves were made in the same way as is described above As parameters of duration the times after injection needed for 50 per cent and 75 per cent of the maximal effect to disappear were calculated and used

The estimation of adrenergic neuron blocking effect

In a few experiments the sympathetic chain on one side was left intact and the ptosis induced by the adrenergic blocking drugs could be studied and estimated in a way analogous to that used for the analysis of sympathomimetic effects

Reliability of the method

To estimate the overall reliability of the method used to measure the palpebral aperture, the variations between occasions' on the decentralized (control) side of 10 individual untreated animals were analysed The mean values (B values, see Fig 4) obtained at each of 5 consecutive occasions from the middle of the whole estimation period were chosen, when the conditions had become stable The variance of these 5 values around their mean was calculated in each animal and an average variance estimated The mean standard deviation between occasions calculated from this average was ±0 24 mm (50 occasions) while the arithmetic grand mean of the aperture size was 2 44 mm

The variance within occasions is mainly due to measurement error To estimate this the variance of individual readings on the denervated and decentralized sides (a, b values respectively see Fig 4 around the means of the occasion A or B) at every one of 5 consecutive occasions were … descending limb of the de… The grand arithmet… nging from 3 0 to 4… n the decentralized s…

Thus with … about 10 per cent to the variance between occasions

Results

The degeneration contraction in untreated rats

On each experimental day a number of rats were selected at random to be studied as controls Their total number in the present investigation was 34 and in every case a degeneration contraction could be observed No significant day to day variation could be proved by an analysis of variation and the control data were therefore pooled Each rat was treated as a statistical unit and the T_{50_a} and the T_{50_d} were found to be 15 29 ± 0 96 and 23 22 ± 1 39 hrs respectively (the mean and standard deviation) The width was 7 93 ± 1 54 hrs and the height (the peak difference) 1 99 ± 0 32 mm The mean curve is shown in Fig 5

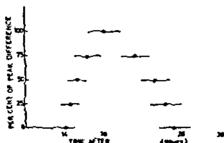


Fig 5 The mean curve constructed from individual curves of 34 normal degeneration contractions in untreated rats. For the method of construction see Fig 4 Means and standard deviation are shown.

There was a slight correlation between bodyweight and T_{50_2} ($r=0.34$, $P<0.05$ and degrees of freedom $=32$) but not between bodyweight and T_{50_3}. The regression coefficient of T_{50_2} in hrs on bodyweight in grams was 0.016 ± 0.008

The effect of phentolamine

4 mg per kg of this reversible alpha receptor blocking agent, dissolved in saline was given i p to each of 5 animals when the degeneration contraction was well developed. Within 20 min after injection the individual differences in palpebral aperture were abolished in all animals but could be reobserved 2—3 hrs later

Preliminary experiments with bretylium

Some preliminary experiments with bretylium were performed in order to see if the findings of Benmiloud and Euler (1963) and Malmfors and Sachs (1965) could be confirmed using our system Two groups of 5 rats received 50 mg per kg bretylium tosylate s c every 8 hrs The rats of one group received their first injection immediately after the operation and thus received 4 doses while the rats of the other group did not receive their first dose until 8 hrs later and thus were given 3 doses In both groups the start of the degeneration contraction was definitely delayed. The delaying effect that is the difference between the mean T_{50_2} of the experimental animals and that of 5 untreated control animals, was 4 40 hrs ($p<0.005$) in the 4-dose group and 2 60 hrs ($p<0.001$) in the 3-dose group However, the difference between T_{50_2} of the two experimental groups is not statistically significant

In these experiments with such high doses bretylium was noted to have a strong sympathomimetic effect. Such an effect during the degeneration contraction proper interferes with the measurements and therefore the effects of single doses of the drug, given well before the start of the contraction were studied in the remaining experiments

Thus a single dose of 50 mg bretylium tosylate per kg was given to two similar groups of rats The animals of one group received their injection at the end of the operation and those of the other group 10 hrs later The delaying effect was 4 25 ($p<0.001$) and 5 30 hrs ($p<0.001$) respectively Again the difference in T_{50_2} of the two experimental groups is not statistically significant

Comparison of the effects of bretylium beta TM 10 bethanidine and BW 392C60 at different doses

The effects on the time course of the degeneration contraction

The drugs were administered by single subcutaneous injections 10 hrs after denervation. The control animals received equal volumes of 0 9 per cent sodium chloride at the same time. The results are shown in Fig 6 Bretylium, even at 1 mg kg caused considerable delay Compared to bretylium the other drugs were much less effective.

There was no significant difference in width (W) or in maximal degeneration

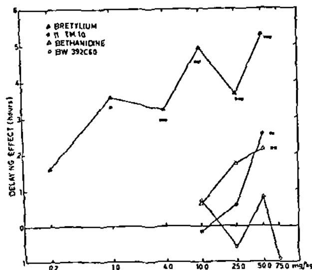


Fig 6 The 'delaying effect' of the drugs at different doses given subcutaneously 10 hrs after denervation The "delaying effect" is measured as the difference between T_{50_2} of the experimental group and that of the control group which was run simultaneously The groups consisted of 5 animals exept at 1 mg/kg bretylium, which had $n=2$ Significance levels xxx = $p<0.001$, xx = $p<0.01$ and x = $p<0.05$

contraction between any of the treated groups and their controls, nor were the slopes of the limbs significantly different Thus the drugs seem to cause a pure delay in the phenomenon

Sympathomimesis

At doses higher than 10 mg per kg, three of the drugs, but not beta TM 10, had a clear and well measurable sympathomimetic action manifested as reversal of the ptosis of both eyes At equal dose levels the sympathomimetic effects were of almost equal duration and even the maximal increase in palpebral aperture was of similar degree (*e g* at 50 mg/kg about 1 6 mm) 75 per cent of the effects had disappeared about 4 hrs after injections of 50 mg per kg

The lack of relation between the 'delaying effect" and neuron blocking activity

In order to study the neuron-blocking action of the drugs simultaneously with the "delaying effect' in the same animal, some experiments were performed in which denervation was made on one side but on the other side the sympathetic trunk was left intact The ptosis induced on the innervated side by the action of the drugs could then be measured and was considered as an effect of adrenergic neuron block The drugs were given 10 hrs after denervation to 2 rats for each drug Bretylium was given at 10 mg per kg and beta TM 10, bethanidine and BW 392C60 at 25 mg per kg All of the drugs caused a marked ptosis In the present connection, however the durations were of special interest These are illustrated in Fig 7 Bretylium differed from the three other drugs The neuron blocking action of bretylium fell to half its maximum almost 3 hrs before the degeneration contraction had reached 50 per cent of its peak while with bethanidine and BW 392C60 the neuron block-

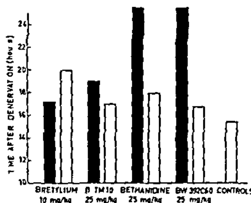


Fig 7 The duration of the neuron blocking effect and the delaying effect estimated simultaneously on the same animal 2 animals were used per drug all injected 10 hrs after denervation Filled columns show the mean of times after injection needed for 50 per cent of the initial value of the palpebral aperture to be reached after the stage of maximal ptosis Open columns show the mean of times after injection needed for the degeneration contraction to reach 50 per cent of its peak In the controls (n=5) this was 15 4 hrs

ing action was of much longer duration and covered the whole time of the degeneration contraction which was delayed little or not at all Beta TM 10 occupied an intermediate position.

Discussion

Ptosis reversal a degeneration contraction

The functional basis of the effect studied is the spontaneous contraction of the sympathetically innervated periorbital smooth muscles which reverses the post denervation enophthalmus and ptosis In mammals there are bundles of smooth muscle fibres unevenly distributed in the periorbital tissue In certain places they are concentrated to form the orbital muscle of Muller the nictitating membrane and small muscles in the upper and lower lids (Muller 1858 1872) The rodents which lack a bony floor to the orbital cavity have a very well developed orbital membrane (muscle) of Muller which covers the defect (Burkard 1902) The adrenergic innervation of the orbital smooth muscles of the rodents including the rat has recently been studied by Ehinger (1966) The different denervation phenomena studied earlier have been shown to be due to release of stored transmitter from the degenerating nerve terminals (cholinergic Emmelin and Stromblad 1957 adrenergic Sears and Barany 1960 Bárány 1962 Coats and Emmelin 1962 Langer 1966 and van Orden *et al* 1967) The character of the degeneration contraction studied in this investigation and the fact that it is blocked by phentolamine indicates that the transmitter release hypothesis can be applied in this case too

Correlation of the degeneration contraction with the results of earlier studies of the postdenervation depletion of catecholamine in the rat

The time course of the postdenervation disappearance of the endogenous catecholamine in different tissues of the rat has been studied with biochemical and histochemical methods by some authors Weiner *et al* (1962) studied the disappearance of noradrenaline from interscapular brown fat bodies and Benmiloud and Euler

(1963) analysed the analogous event in submaxillarly glands with a similar biochemical method Malmfors and Sachs (1965) studied histochemically the changes in catecholamine content and uptake storage mechanisms in the rat iris In all these investigations the catecholamine content in the different organs was virtually unchanged at 8 hrs but barely measurable at 24 hrs after denervation Malmfors and Sachs reported that a large portion of the nerve terminals lost their transmitter between 12 and 16 hrs after denervation In each system of terminals arising from one preterminal axon the catecholamine fluorescence seemed to disappear within one 4 hr period but this happened at times which differed from system to system The biochemical and histochemical findings referred to above correlate well with the functional results of the present investigation The degeneration contraction started approximately 13 to 15 hrs after denervation and reached its maximum about 5 hrs later The phenomenon disappeared somewhat slower and ended about 7 hrs after the peak 23 5 to 26 5 hrs after denervation Malmfors and Sachs found that the depletion proceeded in 2 phases firstly, a rapid phase and secondly a slower one They proposed that the initial and rapid loss was due to deterioration of the pump mechanism of the axon membrane and that the second phase was related to dysfunction of the uptake storage mechanisms of the amine granules Such a two-step release could result in a two step degeneration contraction either visible as a double hump or as a step in the smooth curve No traces of such a phenomenon were seen in the present experiments However this does not rule out a Malmfors Sachs phenomenon since the humps or steps might be smoothed out by temporal dispersion between the course of the degeneration in different axons

The bretylium effect

The adrenergic neuron blocking drugs have repeatedly been reviewed (see for instance Copp 1964 Boura and Green 1965 Mull and Maxwell 1967) Their pharmacology is complex and besides their common neuron blocking action they have other effects in which they differ such as sympathomimesis monoamine depletion and MAO inhibition They also show a variety of actions at cholinergic sites and a capacity for inhibition of the depletion of transmitter from adrenergic nerve endings induced by reserpine and guanethidine again probably different in different compounds As mentioned in the introduction the neuron blocking drugs bretylium and beta TM 10 have previously been shown to delay the denervation phenomenon (Benmiloud and Euler 1963 Langer 1966 Langer and Trendelenburg 1966) The potent neuron blockers used in the present experiments were bretylium beta TM 10 bethanidine and BW 392C60 Besides the fact that two of them had been used for similar studies before they were chosen for comparison because of their difference in structure bretylium and beta TM 10 are quaternary compounds and the other two belong to the benzyl guanidine series see Fig 1 The relative potencies with respect to some of the different actions of three of the drugs are shown in Table I

The delaying effect of bretylium reported by Benmiloud and Euler (1963) and

TABLE I Diagram showing schematically the relative potencies with respect to some of the different actions of three of the drugs used It is based on reports of fairly comparable experiments in rats Beta-TM 10 is excluded because the results reported are not comparable to those of the other drugs It most likely resembles bretylium

Effect	Inhibition of depletion by guanethidine	Direct depletion	MAO inhibition	Sympathomimesis
Bretylium	+	—	+	++
Bethanidine	++	+	+	++
BW 392C60	+++	—	+++	++
References	Costa *et al* 1962	Costa *et al* 1962 Spriggs 1966	Kuntzman and Jacobson 1963 (in vitro) Clarke and Leach 1968	Boura and Green 1959 Boura and Green 1963

+++ = strong
++ = moderate
+ = weak

Malmfors and Sachs (1965) was fully confirmed in the present investigation The delay of the degeneration contraction induced by a single s c injection of bretylium at 50 mg per kg was about 5 5 hrs The greater delay of about 8 hrs observed by Malmfors and Sachs may well be due to the difference in method and possibly dosage.

Langer (1966) reported that beta-TM 10, 10 mg per kg, induced a delay of about 10 hrs in the onset of degeneration contraction in the nictitating membrane of the cat Moreover, he found a significant prolongation of the phenomenon after beta-TM 10 This strong "delaying effect" and the 'prolonging effect" of beta-TM 10 are not quite in agreement with our results We found only a moderate "delaying effect" of this drug even at 50 mg per kg and no 'prolonging effect" of any of the drugs The discrepancy, however, may be the result of species differences

The results of the dose-response experiments clearly indicate that compared to bretylium the other drugs are much less effective in delaying the degeneration contraction in the rat The dose of beta TM 10 and bethanidine needed for a delay of 2.5 hrs was approximately 100 fold that of bretylium The finding that BW 392C60, which structurally closely resembles bethanidine did not give any delay is also remarkable

Bretylium also differed from the other drugs studied in the experiments where the duration of the neuron blocking action and the delaying effect' were simultaneously estimated in the same animal The neuron blocking action of bretylium had already fallen considerably when the degeneration contraction began With bethanidine and BW 392C60, however, the neuron blocking action was of

much longer duration but the degeneration contraction was only delayed little or not at all Thus the greater effectiveness of bretylium in delaying the degeneration contraction is not due to a longer neuron blocking action, and there seems to be no correlation between the neuron blocking action and the 'delaying effect'

The author wishes to express his thanks to Professor E Barany for his unfailing help and encouragement and to Mrs L Bennich Bjorkman, Mrs I Wersall and Mr T Ekwall for skilful technical assistance

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Studies on the Distribution and Elimination of [$^3H_8$] Pyridoxine in Mice

By

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Abstract

Dahlkvist, G, S Lindstedt and H G Tiselius *Studies on the distribution and elimination of [$^3H_8$] pyridoxine in mice* Acta physiol scand 1969 *75* 427—432

[$^3H_8$] Pyridoxine has been administered by i v injection to mice, and the concentration of isotope has been determined in different tissues up to 30 days after the administration An initial high uptake was found in all tissues except brain The concentration decreased rapidly during the first day after administration and then at a slow rate The decrease of isotope concentration was more rapid in liver than in muscle or carcass The elimination of the vitamin from the body reservoir was estimated to be 7 9 per cent per day from day 1 to 30 compared to 6 5 per cent per day as calculated from the rate of excretion of isotope in the urine One week after the administration of the isotope the concentration was approximately the same in heart, skeletal muscle brain and kidney, and 20 % higher in liver Between day 7 and 30 after the administration [$^3H_8$] pyridoxine 75 % of the recovered isotope was found in carcass, 10—15 % in liver and the remainder in other tissues

Few studies have appeared concerning the metabolism of vitamin B_6 Cox *et al* in 1966 reported that 70 % of an injected amount of pyridoxine was rapidly eliminated from rats Johansson and co-workers could follow the excretion of labeled metabolites for more than 3 weeks after the administration of a small amount of labeled pyridoxine to rats (Johansson *et al* 1966 a) and humans (Johansson *et al* 1966 b) and they concluded that the rate of elimination of the vitamin from the body pool was of the order of 1—5 % per day In order to get further information on the fate of administered pyridoxine we have now determined the distribution of isotope in mouse tissues at different times after an i v injection of [$^3H_8$] pyridoxine

Material and methods

Labeled pyridoxine
[^3H, n

Administration of labeled pyridoxine and preparation of tissues

[illegible] intestines were rinsed with water. A small piece of the gastrocnemius muscle was cut out and used as an example of skeletal muscle. Each organ was cut into small pieces which were weighed into small glass vials. These were placed in a desiccator over P_2O_5. The skin was removed from the carcass, which was weighed and then homogenized in a Vir Tis 23 homogenizer (The Vir Tis Co. Inc. Gardiner, New York, N.Y.) in about 50 ml of water. The homogenate was then freeze-dried to a fluffy powder.

Isotope determination

Weighed pieces of the different dried tissues (30—50 mg) or corresponding amounts of freeze [illegible] be ascertained that all vapor had been completely collected. The condensed carbon dioxide was allowed to escape by placing the trap in a dry ice-acetone bath, and the trap rinsed out with 8 ml portions of the fluid used for scintillation counting. This was of the following composition: PPO (2,5-diphenyloxazol) 10 g; dimethyl POPOP (1,4 bis-2 (4 methyl-5-phenyloxazylol) benzene) 0,3 g; toluene 1 000 ml, and methyl cellosolve 600 ml. The samples were counted in a liquid scintillation spectrometer (Packard Instrument Co. La Grange, Ill.) to give a statistical error of less than 5 per cent. The counts for each sample were corrected for quenching by the addition of an internal standard of tritium labeled pyridoxine in water. For each tissue and animal triplicate samples were carried through the procedure. For isotope determination in urine 0.3 ml was added to 16 ml of the counting fluid.

Results

Evaluation of the method for isotope determination

To test the completeness of combustion and the recovery of water in the spiral trap 50 to 200 µl volumes of a standard solution of pyridoxine containing 2,000 cpm per ml were either directly counted in the liquid scintillation counter or added to 50 mg of a freeze-dried homogenate of mouse carcass which was then dried and carried through the combustion procedure. As seen in Fig. 1 a good agreement was obtained between the two methods for isotope assay.

Retention of isotope

Fig. 2 shows the per cent of the injected amount of isotope which remained in the body at different times after the administration as calculated (i) from the actual determinations in the different tissues and (ii) as the difference between the administered amount of isotope and the isotope recovered in the urine in a series of 4 mice. There was a rapid initial loss of isotope from the body during the first day after injection which was followed by a phase with a slow rate of elimination.

The isotope content per mg of tissue in the different organs of the mouse at varying times after the administration of $[^3H_4]$ pyridoxine is shown in Fig. 3. An initially high concentration fell off rapidly within the first day after the injection, whereafter the isotope concentration decreased slowly. In the brain the initial high uptake of isotope was not seen and the maximal isotope concentration was found 1 day after the administration.

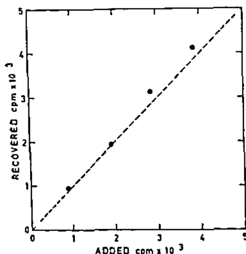


Fig 1

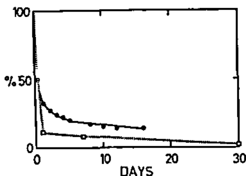


Fig 2

Fig 1 Recovery of isotope in the combustion method Different amounts of [^{3}Hs] pyridoxine in water solution were either counted directly in a liquid scintillation spectrometer *(abscissa)* or added to 50 mg of freeze dried tissue After combustion the isotope in the water was determined by the same method *(ordinate)*

Fig 2 Per cent of isotope remaining in the body at different times after the administration of [^{3}Hs] pyridoxine The solid line has been calculated from the excretion of isotope in the urine of a series of 4 mice and the dotted line from measurements of isotope in the tissues

Distribution of isotope between different tissues

A comparison of the concentrations of isotope in the different tissues 1 and 7 days after the administration is shown in Fig 4 At both times the concentration was highest in the liver followed by heart and skeletal muscle One day after injection a high concentration was also found in the kidney After 1 week the concentration of isotope per mg of tissue was the same in heart and skeletal muscle, brain and kidney and lower in lung intestine and spleen On an absolute basis carcass and liver accounted for 80—90 per cent of the isotope recovered Initially equal amounts of isotope were found in liver and carcass but the relative amount in the carcass then increased and between day 7 and 30 after the injection about 75 % of the recovered isotope was located in the carcass, 10 to 15 % in the liver, and the remainder in other tissues as shown in Fig 5 and 6

Discussion

Previously studies on the in vivo turnover of vitamin B_6 have been performed in rats (Cox *et al* 1966 Johansson *et al* 1966 a) monkeys (Greenberg and Peng 1965) and humans (Johansson *et al* 1966 b Gentz *et al* 1967) Isotope retention curves have been calculated from the excretion of isotope in the urine after the administration of tritium or ^{14}C-labeled pyridoxine and used in a metabolic model to

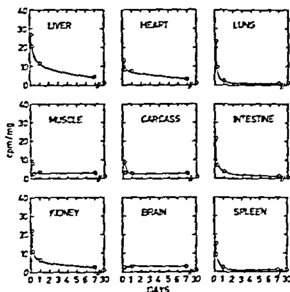


Fig 3 Variation with time of isotope concentration in different tissues of mice after the injection of [$^3H_6$] pyridoxine

estimate the rate of elimination of vitamin B_6 from the body (Johansson *et al* 1966 a, b Gentz *et al* 1967) Necessarily, conclusions drawn from experiments of this type are indirect and have to be based on assumptions on the fate of the injected pyridoxine in the body They should therefore be supported by direct measurements in the tissues We have now chosen mice as experimental animals to make it easy to determine the isotope in organs and tissues Chromatographic separations have established that following the administration of [$^3H_6$] pyridoxine the isotope which is recovered in perchloric-acid extracts of tissues is distributed between the six different forms of vitamin B_6 i.e. pyridoxine pyridoxal, pyridoxamine and their respective phosphorylated forms (Dahlkvist *et al* 1967) A measure of the total vitamin derived from the injected pyridoxine was therefore obtained by direct combustion of the respective tissue Losses by extraction and purification procedures were avoided and it was possible to follow the disappearance of labeled derivatives of vitamin B_6 over a long period of time.

The course of the excretion of isotope in the urine of mice after the administration

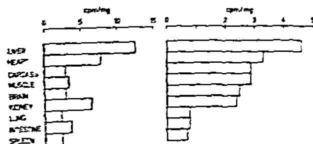


Fig. 4 Isotope concentration in different tissues of mice at different times after the injection of [$^3H_6$] pyridoxine at 1 day (*left*) and 7 days (*right*) after the injection.

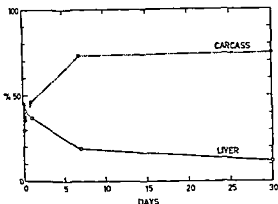


Fig 5

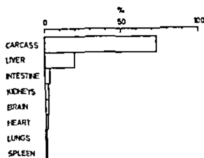


Fig 6

Fig 5 Per cent of total amount of recovered isotope in liver and carcass 1 to 30 days after the injection of [$^3H_8$] pyridoxine

Fig 6 Per cent of total amount of recovered isotope in different organs of mice 7 days after the injection of [$^3H_8$] pyridoxine

of labeled pyridoxine resembled that found in rats and humans, i e an initial rapid excretion followed by a slow elimination (Fig 2) The slope of the second phase of the isotope-retention curve calculated from excretion data was 0 065 day^{-1} *i e* slightly higher than found previously in rats, but in accordance with the generally high metabolic rate in mice The loss of isotope from the body during the first 2 days after administration was high Previous studies in rats have clearly demonstrated that the magnitude of this initial excretion is dependent on the amount of pyridoxine injected, in this series 2 μg by the intravenous route The isotope retention curve obtained by subtracting the excreted amount of isotope from the administered amount should be compared with that drawn from results of direct measurements of isotope remaining in the body at different times after injection of [$^3H_8$] pyridoxine The two curves run almost parallel, the slope of the second phase of the curve drawn from direct measurements is 0 079 day^{-1} After 1 week about 10 % of the injected amount of isotope was recovered in the body by direct measurements but 20 % calculated to remain on the basis of excretion values It is however technically difficult to obtain quantitative collection of mouse urine and even a small absolute loss during the first collection period would tend to displace the isotope retention curve upwards whereas incomplete recovery of isotope from the whole animal would have the opposite effect

The variation of isotope concentration with time was similar in the different tissues All with the exception of brain showed initially a high concentration which rapidly decreased and then declined more slowly between day 1 and 30 The absence of the initial high uptake in brain may possible be explained by a low permeability of the blood brain barrier for the injected pyridoxine From Fig 3 it is apparent that there were some variations in the rate of elimination from the different

tissues Thus, the loss of isotope from liver was faster than from muscle or carcass An approximate estimate of the slopes of the elimination curves for carcass and liver gave the values 0 053 day $^{-1}$ and 0 087 day $^{-1}$, which should be compared with the above mentioned figure of 0 079 day $^{-1}$ for the whole animal The slope of this part of the isotope retention curve may be taken as an approximate measure of the rate of elimination of the vitamin from the storage compartment of a two-compartment system (Johansson *et al* 1966 a, b)

The highest initial concentration was found in the liver, which after 15 minutes *contained 40 % of the total recovered isotope Between day 5 and 30 about 75 %* of the recovered activity was located in the carcass, 10—15 % in the liver and the remainder in other tissues It is of interest in this connection that Krebs and Fischer (1964) have estimated that over 50 % of the total amount of vitamin B_6 in the body occurs as pyridoxal 5 phosphate bound to phosphorylase In a study on the distribution of vitamin B_6 in mice Lyon *et al* (1962) have reported concentrations of vitamin B_6 of about 400 µg per g of nitrogen in liver and 150 to 200 µg per g of nitrogen in muscle in two strains of white mice in agreement with the higher concentration of isotope in liver observed in this study

The present study has thus verified that part of an administered amount of pyridoxine is retained for a long period of time in the body and is eliminated with a half life of about 10 days It has also shown that the concentration in the tissues reaches approximately the same value after an initial period of equilibration The rate at which the administered pyridoxine is converted to the active forms of the vitamin in the different tissues remains to be studied

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From the Neurosurgical Clinic, Helsinki, Finland

Electrical Stimulation of a Human Ventrolateral-Subventrolateral Thalamic Target Area

I. The stimulation method and the application of repeated stimulus trains of constant composition

By

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Received 20 June 1968

Abstract

JOHANSSON, G G *Electrical stimulation of a human ventrolateral-subventrolateral thalamic target area I The stimulation method and the application of repeated stimulus trains of constant composition* Acta physiol. scand 1969 75 433—445

A method is described for electrical stimulation of human subcortical brain structures The necessity is shown for monitoring both the voltage across and also the current through the stimulated brain tissue, as well as the duration of the stimuli applied Stimulus trains of constant composition were repeatedly applied to a ventrolateral subventrolateral thalamic target area in 12 alert patients undergoing stereotaxic thalamotomy Stimulation at the clinical threshold resulted in motor and sensory responses projected to contralateral parts of the body In spite of small temporary variations, no distinct change of response from one stimulus train to the next in the same patient could be detected when the stimulation was applied at intervals of either 15, 30, or 60 sec The current thresholds on the other hand, showed a tendency to decrease if the intervals were less than 60 sec The stimulation method and the results of stimulation are discussed

The functional anatomy and physiology of the human brain can be studied by electrical stimulation Electrical stimulation is also used by the neurosurgeon as an aid to localization Especially in stereotaxic neurosurgery, in which the surgeon is working inside a "closed skull" it is important to carry out stimulation before producing an irreversible operative lesion Unexpected responses warn the surgeon that the electrode is not situated in the intended brain structure

The results of electrical stimulation reported by different investigators have often been inconsistent One of the most obvious reasons for this is the considerable anatomical variability in brain structure between different individuals (*cf e g* Brierley and Beck 1959, Schaltenbrand and Bailey 1959 and Van Buren and Maccubin 1962) This variability makes it difficult to determine the exact position of the

stimulating electrode in the brain. In animal experiments, uncertainty can be eliminated by postexperimental histological examination. But when stimulating the human brain one has to rely on the statistical anatomy offered by stereotaxic brain atlases

A second factor that might cause inconsistent stimulation effects, though more difficult to control and study, is spread of the current (*cf e g* Phillips 1956, Hern, Phillips and Porter 1962 and Landau Bishop and Clare 1965) As a result of such spread, responses might be elicited from brain structures with low stimulation thresholds situated at some distance from the site of the electrode.

The effects of stimulation have repeatedly been shown to be dependent on the stimulus parameters in both animal and human studies (*cf e g* Wyss and Obrador 1937, Hines 1940, Hare and Geohegan 1941, Berry, McKinley and Hodes 1942, Delgado and Livingston 1948, Liddell and Phillips 1950 and 1951, Moruzzi 1950, Hyde and Gellhorn 1951, Kaada 1951, Lilly, Austin and Chambers 1952, Cure and Rasmussen 1954, Mihailovic and Delgado 1956, Ervin and Mark 1960, Buchwald *et al* 1961, Gengerelli 1961, Hassler 1961, Hassler and Riechert 1961, Bogach and Kosenko 1964, Libet *et al* 1964, Bergström *et al* 1966 and Traczyk and Jakubowska 1966) Therefore a third important way in which misunderstandings may arise concerning the stimulation responses is constituted by incompletely reported stimulus parameters

At the Neurosurgical Clinic in Helsinki a ventrolateral subventrolateral thalamic target area was stereotaxically electrocoagulated through a metal electrode for the treatment of Parkinson's disease, hereditary intention tremor and certain other brain diseases. Electrical stimulation performed prior to the coagulation provided an opportunity to study the effects of varying some stimulus parameters on the clinical responses elicited under almost physiological conditions and without much extra strain for the patients

In this first part of the investigation the methods used for surgery and stimulation are reported. Further, the brain structures stimulated are defined. Finally, it is shown that repeated application of stimulus trains consisting of electrical pulses of constant repetition frequency and duration elicited clinical threshold responses of relative constancy

Methods

Surgery[1] and target area for stimulation

Stereotaxic surgical intervention was performed with a Mundinger—Riechert stereoencephalotome. Operative premedication consisted of an i m injection of 5 mg perphenazine (Trilafon®) and 50 mg cyclizine lactate (Marzine®) given about 2 hrs prior to electrical stimulation. Local Citanest Exadrin® anesthetic was used when the stereoencephalotome was being screwed to the cranium with 4 metal screws. The same anesthetic was also used for the skin incision. The total amount of 1 % Citanest Exadrin® used was about 15 ml for each patient.

Through a frontal burr hole situated on the coronal suture or slightly anterior to it, 3—5 cm lateral to the sagittal suture the lateral ventricle was cannulated with a polyethylene catheter Through this catheter about 25 ml of air was insufflated. The midline of the third

[1] The surgery was carried out by L. V Laitinen, M D

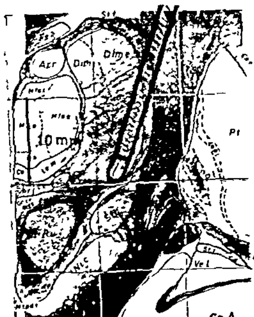


Fig 1 Frontal section of the brain, 4 mm posterior to the midpoint of the *intercommissural line, showing the approximate position of the electrode* The vertical double line on the left of the figure denotes the sagittal plane through the midpoint of the third ventricle and the horizontal double line denotes the plane passing horizontally through the intercommissural line The electrode was drawn to scale on the

1959)

Abbreviations for Fig 1 and 2

B co s = Brachium colliculi superioris, Cd = Caudatum, Ce pc = Nucleus centralis parvocellularis, Ci p = Capsula interna, crus posterius, F istth = Fasciculus interstitio-thalamicus, H₂ = Campus Foreli H₂, Li por = Nucleus limitans portae, Ni = Niger Ps pd = Pes pedunculi, Pt = Putamen, Pu = Pulvinar thalami (nucleus posterior
Rt = Reticulatum thalami, Rt im = Nucleus reticula
subthalamicum, Str pd i = Stratum pedunculi intern
ventrocaudalis anterior internus V c i = Nucleus
V m e = N
Nucl
z c = Nuclei zonae incertae caudalis Z im.e =

ventricle and the anterior and posterior commissures, visualized on X-ray pictures, were used as reference points for the calculation of the target area. Through the same burr hole an electrode was inserted into the target area, lying on the intercommissural line, 11 5—13 0 mm lateral to the midline of the third ventricle and 16 0—17 0 mm posterior to the anterior commissure During the introduction of the electrode the brain tissue impedance was measured through the roving electrode by means of a Wheatstone bridge (Laitinen, Johansson and Sipponen 1966) This was performed in order to locate the margins of the thalamus and the internal capsule When the electrode had been inserted, 1 or 2 short electrical stimulations (60 square wave pulses/sec, a pulse duration of 1 msec and a slightly supraliminal peak current were performed in order to make sure that the electrode tip was not lying in an undesired structure After these preliminary tests the studies to be described in this investigation were carried out Finally an electrocoagulative lesion was produced through the same electrode by means of a thermocontrolled Coagrader high frequency coagulator (N V Vitatron Amsterdam)



Fig 2 Sagittal section through the thalamus and adjacent structures 11 mm lateral to the midline of the third ventricle with the electrode placed in the approximate stimulation target area. The horizontal double line denotes a horizontal plane passing through the intercommissural line The vertical double line denotes a frontal plane through the midpoint of the intercommissural line (Modified from Schaltenbrand, G and Baily, P Introduction to Stereotaxis with an Atlas of the Human Brain Stuttgart Georg Thieme Verlag 1959)

The stimulation circuit

The stainless steel electrode was insulated with porcelain and manufactured by Vitatron, Amsterdam (Van den Berg and van Manen 1962) The uninsulated tip of the monopolar electrode was 2 8 mm in length and 2 mm thick The surface area of the tip was approximately 18 mm² During stimulations this electrode was cathodal with respect to the indifferent, remote electrode, which consisted of 2 plates of stainless steel attached by means of steel springs between the neck of the patient and the stereoencephalotome The total area of these plates was 25 cm² Before the plates were attached, the skin was scrubbed with alcohol and the plates coated with electrode jelly The four insertion screws of the stereoencephalotome contributed further to the indifferent electrode

The unidirectional square wave pulses were applied with a Grass Model S 4 stimulator through a Grass SIU B stimulus isolation unit (Fig 3) The patient was carefully insulated from the ground to avoid any leakage of current Between the stimulator and the patient 2 serial resistances, of 10 and 1 (±1 per cent) kilo-ohms respectively, were connected Together they were enough to maintain for measurements in these experiments, a good "constant current" output of the stimulator The maximum peak current output of the circuit during stimulation was about 10 mA The voltage drop across the standard resistance of 1 kΩ and the voltage drop across the patient were both monitored differentially on a Tektronix 402 A cathode ray dual beam oscilloscope The voltage drop across the standard resistance, which was separated from the patient by an unshielded cable 1 3 m long, was used for calculation of the current. The rise time of the pulses over the standard resistor, as shown on the oscilloscope, was 11 μsec The rise time of the voltage pulses across the patient was dependent on the duration of the pulses, i.e. within the range of durations tested the voltage rose with the pulse duration (Fig 4) Thus the peak voltage/peak current ratio changed when the pulse

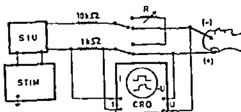


Fig 3 Diagram of the stimulation circuit The variable resistor R was used as a substitute for the patient whenever the values of the stimulus parameters were set

duration was changed from one value to another This ratio for different pulse durations, recorded in 15 patients with Parkinson's disease during electrical stimulation of the ventrolateral-subventrolateral thalamic target area, is illustrated in Fig 5 Besides increasing with increasing pulse duration, the peak voltage/peak current ratio also varies greatly in different patients

With pulse repetition frequencies ranging from 1 to 200/sec and with other stimulus parameters unchanged, the appearance of the pulses did not change

An increasing peak current (current density), studied in 5 Parkinsonian patients, caused a decreasing peak voltage/peak current ratio, as illustrated in Fig 6 The decrease of the ratio within the range of current densities tested, is shown to be maximally of the order of 10 to 15 per cent

The stimulation procedure

At the time (10—11 a m) when the stimulations were performed the patient had been at least 60 min in the operating theatre He lay on his back with his head rigidly screwed to the stereoencephalotome The head, neck, upper extremities and feet were uncovered The other parts of the body were not exposed The average temperature in the operating theatre was [illegible]

whenever the values of the stimulus parameters were set for the next stimulus train Unnecessary stimulation of the patient could thus be avoided When the desired values had been set, the patient circuit was switched on and one of the parameters, peak current or pulse duration was rapidly increased until clinical responses could be observed (first phase of stimulation) When the responses had been identified after a pause of at least 1 min a second stimulation was performed Now the variable parameter was continuously decreased from the supraliminal value just achieved until all observable responses had vanished (second phase of stimulation) The train duration could not be kept constant with this technique, it varied between 5 and 10 sec The last clinical responses irrespective of their projected [illegible]

the first phase of stimulation

The number of stimulus trains applied varied from 5 to 15 for different patients depending on the condition of the individual patient and his ability to cooperate

Questioning for subjective sensations

In the operating theatre prior to performance of the series of stimulations, the patient was told that from time to time he might experience some sensations somewhere in his body during the following 10 to 20 min The subjects were also told that possible sensations were due to the application of weak electric currents to his brain The patient was further told to announce the onset and cessation of a sensation but nothing else unless asked To achieve and to maintain as uniform a state of alertness as possible the patient was asked to keep his

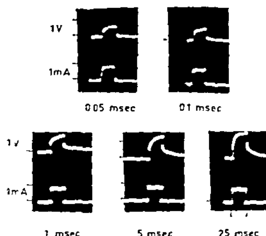


Fig 4

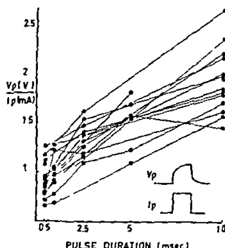


Fig 5

Fig 4 Voltage drop across and current through the brain of a Parkinsonian patient who was electrically stimulated with pulses of different durations The pulse repetition frequency was kept constant at 10 sec

Fig 5 Peak voltage (V_p)/peak current (I_p) ratio for pulse durations ranging from 0 5 to 10 msec studied in 15 Parkinsonian patients. The peak current was kept constant at 0 5 mA and the pulse repetition frequency at 10/sec.

eyes open throughout the series of tests. During the first phase of stimulation the subject had an opportunity to consider the nature of the sensation He was asked to describe in his own words what he experienced and where in the body the sensation was felt If the patient's report was not clear to the observers, additional leading questions about the nature and localization of the sensation were asked Concerning the nature of the sensation several alternatives were presented to him in such cases, i e he was asked whether the sensation felt like pain warmth, movement, heat, numbness, tingling, electricity, etc He was also asked if he [illegible] it by telling [illegible] was then ask [illegible] the ones you experienced previously? Further, he was asked whether the localization of the sensation was the same as before or if it had changed. The reliability of the patient's reports was checked from time to time by performing a dummy test without a stimulus but with the same preceding remarks or by real tests not announced to the subject in advance Only subjective sensations which were thought to be the result of direct stimulation of sensory neuronal elements were recorded Thus no subjective sensations elicited by tremor or by movements evoked by the stimulus were recorded The subjective sensations, in this restricted sense, will in the following be called "sensory responses

Patients

The material for the investigation consisted of patients operated upon stereotaxically for hyperkinetic disorders from July 26, 1966, to Sept 20, 1967 Patients who were uncooperative or in poor condition were not included in the study

The total number of patients was 65 Fifty two of them suffered from Parkinson's disease and 10 had a hereditary intention tremor The remaining 3 patients suffered from choreo-athetosis, atypical congenital tremor and Wilson's disease respectively There were 36 men and 29 women Five of the patients were between 24 and 29 years old The mean age of the remaining 60 patients was 57 (SD ± 7) years, the oldest patient being 70

The patients were admitted to hospital 1—2 days prior to surgery All antiparkinsonian or sedative drug therapy was withdrawn on admission.

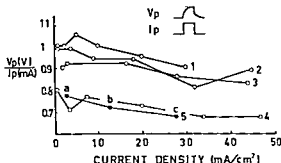


Fig 6 Peak voltage (V_p)/peak current (I_p) ratio for different current densities at the electrode tip studied in 5 Parkinsonian patients The pulse duration was kept constant at 0 5 msec and the pulse repetition frequency at 10/sec

Stimulation data from 28 of the patients are presented in more than one part of the present investigation. Therefore the patients participating in each different group of experiments will be presented in greater detail in the relevant part

The effects of insertion of the electrode on Parkinsonian tremor

The preoperative Parkinsonian tremor stopped immediately in about 60 per cent of the patients as soon as the electrode had been inserted into the stereotaxic target area. In some further [illegible] of the [illegible] to appl[illegible] tremor [illegible] the stimulus was applied A small group of patients had a slight or moderate continuous tremor or occasional tremor bursts during the experiments The basis for the stimulations and also for the interpretation of the effects was therefore not quite the same in all patients However it seemed to be very difficult to exclude patients on grounds of such often temporary and slight differences in the degree of tremor present and therefore all patients stimulated were included in the series

Results

Constancy of the clinical threshold responses elicited by repeated application of stimulus trains of constant composition

5 to 8 stimulus trains, composed of pulses with a constant duration and repetition frequency were applied at regular intervals to the stimulation target area of each of 12 patients The composition of the stimulus trains had been chosen quite arbitrarily and varied from patient to patient

According to the stimulation technique described one stimulus parameter in these particular experiments the peak current, was readjusted during every single stimulus train to keep the stimulation at the threshold for clinical responses

The patients the composition of the stimulus trains applied to each of them and the threshold responses elicited are shown in Table I

The general features of the clinical motor responses elicited did not change from

TABLE I Patients and clinical threshold responses obtained by electrical stimulation of the ventrolateral - subventrolateral thalamic target area Abbreviations P = Parkinson's disease, Trh = *hereditary intention tremor*, Wi = *Wilson's disease*

Patient no	Age	Sex	Diagnosis	Brain hemisphere stimulated	Train repetition frequency (trains per minute)	Pulse repetition frequency (pulses per second)	Pulse duration (msec)	No of repeated stimulus trains applied
1	62	F	P	Right	1	1	1	5
2	55	M	Trh	Left	1	20	2 4	5
3	68	M	P	Left	1	1	10	5
4	55	F	P	Left	1	20	2 8	5
5	21	F	Wi	Left	1	20	2 5	5
6	64	F	P	Left	1	20	3 1	5
7	46	F	P	Right	1	20	4	5
	64	F	Trh	Left	1	20	2 4	5
9	64	M	P	Right	2	10	10	7
10	60	M	P	Left	2	10	10	8
11	65	F	P	Left	4	10	10	8
12	44	M	P	Left	2	10	10	7

one stimulus train to the next If, for example, the response at the first stimulus train was projected to the contralateral hand, it remained there at all stimulations No new parts of the body were involved at the stimulation threshold with repeated stimulus trains On the other hand, small local variations in the number of muscle units involved and in the strength and type of movements were occasionally observed during the course of the same stimulus train and also from one train to the next *This was most significant in the patients in whom the pulse repetition frequency* was kept at 1/sec (patients no 1 and 3)

The "sensory" responses elicited also remained unchanged This concerned both their character and their projected localization in the body Patient no 5 constituted the only exception This patient, however, was a very sensitive young woman with some degree of psychic instability which perhaps explains the exceptional responses

The time intervals between the different stimulus trains were kept at different lengths (*i e* 15, 30, or 60 sec) for different patients in order to study whether the constancy of the responses depended on the length of the interval Within these time ranges this seemed not to be the case The threshold peak current tended to decrease continuously, however, if the interval was less than 60 sec (Fig 7) This was especially obvious when the criterion for the threshold values was "sensory" responses reported by the patients (patients no 11 and 12)

Clinical responses at the stimulation threshold
Variable jerks of the contralateral thumb and flexion jerks in the elbow of the same extremity.
Subjective sensation of electricity in the neck, tremor in the jaw and in the contralateral hand
The first 3 stimulus trains elicited muscular twitches in the lips The strongest effect seemed to be homolaterally in the upper lip The following 2 trains elicited twitches only contralaterally
Subjective sensation of electricity in the contralateral hand and arm; tremor in the contralateral arm
The first two stimulus trains elicited subjective sensations of electricity contralaterally in the face and hand The following train resulted in "pain through the head" and finally during the last 2 stimulus trains the patient reported a sensation "like salt coming out of her nose"
Subjective sensation of electricity in the upper lip and in the contralateral hand
Subjective sensation of warmth in the throat and in the contralateral hand
Subjective sensation of tremor in the contralateral hand
Tremor in the lower lip
Tremor contralaterally in the lower lip
Subjective sensation of electricity contralaterally in the face and in the arm with all stimulus trains
Subjective sensation of tremor in the lower lip

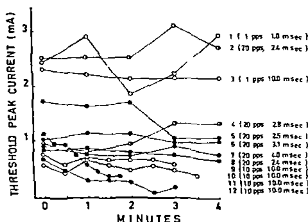


Fig 7 Threshold peak currents of the clinical responses obtained by repeated application of stimulus trains to ventrolateral subventrolateral thalamic structures of the patients listed in Table I The circles along the curves denote the stimulus trains An open circle signifies that the threshold responses during the train were motor and a filled circle that they were "sensory" in character Responses which, at the stimulation threshold, were both "sensory" and motor in character are denoted by the half filled circles The number *after each curve is* the patient's number (see Table I) The repetition frequency and the duration of the stimulating square wave pulses are given in brackets after the patient's number

Discussion

The importance of monitoring both the current through and also the voltage drop across the brain tissue stimulated was pointed out by Mickle (1961) The reasons were made abundantly clear by the papers of Rowell (1963) and Weinman and Mahler (1964) Rowell showed that when small stainless steel electrodes were used, the current flow through saline and insect brains showed wide variation for different pulse repetition frequencies and lesser variation of wave form Weinman and Mahler (1964) showed that at the application of square wave pulses the peak voltage/peak current ratio did not behave linearly at the electrode electrolyte (saline) interface This nonlinear behaviour was especially significant for different pulse durations and current densities Within certain limits the pulse repetition frequency did not affect the ratio for most electrodes The behaviour in brain tissue of the steel electrode described in this study did not essentially differ from the behaviour of the steel electrodes tested by Weinman and Mahler (1964) in saline. However electrodes of different sizes and materials behave in different ways (Weinman and Mahler 1964, Weinman 1965) Consequently these facts constitute important reasons for the simultaneous monitoring of both the voltage drop and the current flow and also the duration of the pulses applied during electrical brain stimulation.

A further important reason is the individual variability of the brain tissue impedance. In the present study this variability was indicated by the differences of the peak voltage/peak current ratio in different patients

All experimental studies concerning the human being especially those performed in an operating theatre are attended with many problems including shortage of time, sterility and patient safety problems lack of control series ethical considerations, etc These are general problems which arise everywhere where such experiments are performed and need not to be discussed here One or two problems were specific to this study The patients were elderly people with Parkinson's disease often combined with some mental deterioration. The antiemetic and tranquilizing drugs cyclizine and perphenazine used for premedication probably caused a mild drowsiness in some patients and thus a decrease in alertness which may have increased the stimulation thresholds Communication between the observers and the patient was for these reasons very likely not optimal in some cases In addition, stimulation responses occurring in the unexposed trunk may have escaped the attention of the observers Further some of the "sensory" responses may wrongly have been excluded from the results in cases where they existed together with strong motor responses and conversely some of the "sensory" responses may have been due to subclinical muscle activity

The clinical threshold responses did not differ significantly whether ascending or descending currents were employed. The reason for choosing the descending thresholds as recording points was that it seemed easier to decide when a given response just vanished than when it started. This was especially apparent for the "sensory" responses reported by the patients themselves

The train duration, with the stimulation technique used, varied from 5 to 10 sec Using another technique, i e applying trains in which the peak current (or pulse duration) of the pulses was decreased stepwise prior to each individual application (instead of continuously during the application of the stimulus train), the train duration could have been kept quite constant Such a procedure would have required the application of many stimulus trains for every single parameter combination in order to determine the stimulation threshold Shortage of time precluded this probably slightly more reliable technique The nonconstant train duration may, however, lay the experiments open to criticism It has been shown that both the thresholds and the stimulation responses are dependent on the length of the stimulus train when the human somatosensory cortex or postero-ventrolateral nucleus of the thalamus is stimulated (Libet *et al* 1964) However, this concerns mainly only train durations of less than about 1 sec Lilly, Austin and Chambers (1952), who stimulated the motor cortex of cats and dogs, reported a uniform fall in current threshold when they increased the duration of the trains from 0 1 to 4 sec and in one case to 20 sec In the present investigation it was impossible to notice any appreciable difference of the stimulation thresholds or of the clinical responses when stimulus trains of either 5 or 10 sec were applied

In the present experiments it was shown that no tendency for a change from motor to "sensory' responses or vice versa could be observed on repeated stimulation of the ventrolateral subventrolateral thalamic target area with stimulus trains of constant composition Neither did the main localization of the projected threshold responses in the body change Though not quite comparable as regards the time intervals between stimulations, the findings are in agreement with the results reported by Ervin and Mark (1960) They stimulated human thalamic structures through implanted electrodes and concluded that with fixed stimulus parameters the responses were quite consistent from day to day

The small variations of the responses on a more local level and also the irregular variations in the current thresholds were presumably partly due to changes in the alertness of the patients during the experiments Further the state of activity of the tissues in the vicinity of the electrode tip at the moment when the stimulus was applied was probably of importance in this respect This small variability of the responses is very likely similar to the instability of the responses evoked by stimulation of the motor cortex as already reported by Brown and Sherrington (1912)

Conclusions

The voltage drop across and the current through the patient, together with the duration of the pulses applied, were all monitored during electrical brain stimulation It was demonstrated that this was necessary in order to obtain adequate information about the stimulus parameters and the electrical behaviour of the stimulating electrode

The brain tissue was stimulated by means of a stimulator with a constant cur-

rent' output through a steel electrode The ratio between the peak voltage and the peak current of the square wave pulses applied varied for different pulse durations and to a slighter extent for different current densities

On stimulation of the ventrolateral subventrolateral thalamic target area the peak voltage/peak current ratio showed considerable variations in different patients

Electrical threshold stimulation of the target area was performed with 5 to 8 *repeated stimulus trains The trains consisted of pulses of constant* duration and repetition frequency The stimulations resulted in motor and sensory' responses projected to contralateral parts of the body In eleven out of twelve patients application of the trains did not result in any appreciable change, from train to train of *the clinical responses elicited*

The threshold peak current showed a slight variation from one stimulus train to the next but did not exhibit any tendency to change systematically if the intervals between the trains were kept at 60 sec at least

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From the Neurosurgical Clinic, Helsinki, Finland

Electrical Stimulation of a Human Ventrolateral-Subventrolateral Thalamic Target Area

II. Combination of different durations of single pulses with the peak current just adequate to evoke clinical responses

By

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Received 20 June 1968

Abstract

JOHANSSON G G *Electrical stimulation of a human ventrolateral-subventrolateral thalamic target area II Combination of different durations of single pulses with the peak current just adequate to evoke clinical responses* Acta physiol. scand. 1969 75 446—455

A ventrolateral-subventrolateral thalamic target area was electrically stimulated in connection with stereotaxic neurosurgery in 20 alert patients with Parkinson's disease, in 5 patients with hereditary intention tremor and in 1 patient with an atypical congenital tremor The purpose [illegible] every pulse duration tested with the peak current. The responses consisted mainly of muscular jerks or twitches projected to various contralateral parts of the body The localization of the threshold responses depended on the pulse duration used. The motor character of the responses remained unchanged with all pulse durations tested. The peak current pulse duration relationship for the threshold responses was studied. The results are discussed.

In a previous study the technique used for electrical stimulation of a ventrolateral-subventrolateral thalamic area in human subjects in connection with stereotaxic surgery was described (Johansson 1969

The effects were also shown of repeated application to the target area of stimulus trains consisting of square wave pulses with a constant duration and repetition frequency The repeated stimulation did not result in any significant change from train to train of the projected clinical threshold motor and sensory responses evoked.

In the present study the clinical responses elicited by stimulation of the same target area were further investigated. Pulses of different durations were combined with the minimal peak current adequate to evoke threshold responses The pulse repetition frequency was kept constant at one pulse/sec The peak current—pulse duration relationship at the threshold for the responses elicited was also studied and will be discussed.

Methods

The surgical and stimulation methods have been described in detail previously (Johansson 1969)

The stereotaxic target area, which was the same as before (Johansson 1969) was situated ... the third ventricle and ... site presumably cor ... nclature in accordance ... amus ... lirectional square wave pulses were applied through a monopolar porcelain insulated stainless steel electrode which was cathodal with respect to an indifferent remote electrode The bared tip of the electrode was approximately 18 mm² The current through and the voltage drop across the patient were monitored and recorded

The stimulations were performed with pulses of durations ranging from 0 1 to 25 msec The pulse repetition frequency was kept constant at 1/sec The train repetition frequency was 1/min or slower and the duration of the stimulus trains varied from 5 to 10 seconds According to the stimulation technique used (Johansson 1969), the peak current was increased during the first stimulus train until the stimulation resulted in clinical responses At the second stimulus train the peak current was continuously decreased from the slightly supraliminal value just achieved The last clinical responses which could be recorded in this way regardless of whether they were motor or sensory in character or of their projected locali ...

2.5—5 0 and 10—25 msec, respectively

Patients

Twenty-six alert patients were stimulated in connection with stereotaxic neurosurgery—one of them bilaterally Twenty of the patients suffered from Parkinson's disease 5 had a hereditary intention tremor and 1 had an atypical congenital tremor The last mentioned patient was a 27 year-old man and the patient who was operated upon and stimulated bilaterally was a 28-year-old housewife with hereditary intention tremor The age of the other 24 patients varied from 42 to 70, the mean age being 56 (SD±7) years There were 13 men and 13 women Two of the patients had previously undergone a stereotaxic operation on the opposite side of the brain. One patient had previously had a stereotaxic operation on the same hemisphere but in a slightly different target area In 10 of the patients stimulations were carried out in the right and in 17 in the left hemisphere

Results

All the responses described in the text and shown in the tables were contralateral to the brain hemisphere stimulated unless otherwise stated

I Character of the responses

The threshold responses evoked by the stimulations were mostly motor in character

With a few exceptions, the motor responses consisted of muscular twitches or jerks in different parts of the contralateral half of the body They were more or less synchronous with the stimulus pulse Sometimes the pulses, especially those with a duration shorter than 1 msec resulted in twitches at the stimulation threshold which were irregular and of different strengths With pulse durations longer than 1 msec, the twitches were mostly synchronous with the stimulus pulse and of a rather constant strength for a given peak current applied In those few patients with a tremor

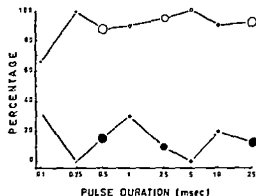


Fig 1

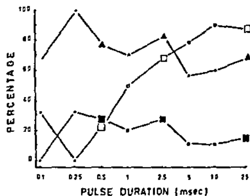


Fig 2

Fig 1 Percentages of patients from Table I presenting "sensory" (filled circles) and/or motor (open circles) threshold responses The size of the circles indicates the relative number of patients stimulated at a given pulse duration The pulse duration axis is logarithmic

Fig 2 Percentages of patients from Table II presenting threshold responses projected to different parts of the contralateral side of the body The symbols denote percentages of patients with responses projected to the upper extremity (triangles), the head neck (open squares) and the lower extremity (filled squares) The changes in the percentages of patients presenting responses projected to the head neck region is highly significant ($P<0.001$) The size of the symbols indicates the relative number of patients stimulated at a given pulse duration The pulse duration axis is logarithmic

in the extremities during stimulation, the stimuli modulated the tremor, *i e* the tremor was superposed by jerks corresponding to the pulses applied

During stimulation at one or more pulse durations 6 of the patients reported that they felt twitching or pulsatile sensations in the contralateral side of the body These sensations could not be related to any visible or palpable motor activity at the threshold stimulation and were therefore considered to be due to direct stimulation of sensory neuronal elements They were called "sensory" responses (see also Johansson 1969) In 3 of the patients however, the sensations could be transformed into motor jerks by increasing the peak current or the pulse duration to supraliminal values

Sensations of muscular twitches, jerks or tremor reported by patients with such motor activity simultaneously visible in corresponding parts of the body were not included under the term "sensory" responses

Table I shows the number of patients in whom at the stimulation threshold motor, "sensory" or both types of responses were simultaneously evoked In some of the patients the stimulation with pulses of a duration of 0 1 to 0 5 msec did not evoke responses at all, even when the maximum peak current (about 10 mA) obtainable was applied

In Fig 1 the percentages of patients from Table I with "sensory" and/or motor responses, respectively, are shown for the different pulse durations The above mentioned negative stimulations were not taken into consideration in calculation of the percentages Motor responses were recorded in about 90 per cent and "sensory"

TABLE I Number of patients presenting motor and/or "sensory" threshold responses on stimulation of the ventrolateral subventrolateral thalamic target area One patient is included in the table twice, because she was stimulated bilaterally The duration of the pulses applied varied from 0.1 to 25 msec and the threshold peak current correspondingly from 10 to 0.6 mA The pulse repetition frequency was 1/sec

Pulse duration (msec)	0.1	0.25	0.5	1	2.5	5	10	25
Total number of patients stimulated	8	8	27	10	22	9	10	26
Number of patients who reacted to stimulation with clinical responses	3	3	26	10	22	9	10	26
Number of patients who reported 'sensory' responses at the stimulation threshold	1	—	4	3	2	—	2	3
Number of patients who reacted with motor responses at the stimulation threshold	2	3	23	9	21	9	9	24

responses in about 10 per cent of the patients These percentages remained statistically[1] unchanged with all pulse durations tested

II Projected localization of the responses

Sometimes it was possible to identify a certain muscle as the exact site of excitation Mostly the motor response was not so well localized Then the projection site was recorded as the hand, arm leg, etc

The lips and the corner of the mouth were sites to which motor responses were frequently localized

With some of the pulse durations tested, 5 of the patients showed motor responses elicited from the orbicularis oculi or palpebral muscles In 2 of these patients the responses were homolateral and in 1 bilateral

Of the hand muscles involved, the thenar and interosseus muscles were the ones most often affected The arm and leg responses were difficult to relate to the particular muscles involved

The few sensory responses reported were situated in the hand arm mouth and in 1 of the patients (no 17 Fig 3) deep in the head

The localization of the projected responses was mostly not the same when long and when short pulses were applied The body region from which stimulation responses could be evoked was divided into 3 parts 1) the head and neck, 2) the upper extremity and 3) the lower extremity The distribution of the patients according to the body part to which the threshold responses were projected, regardless of whether these were sensory or motor in character or both simultaneously, is given in Table II As the pulse duration increased, the simultaneous involvement of two or even all three areas became more usual In Fig 2 the percentages

[1] The statistical analysis was performed by solving the regression equation $Y = a_0 + a_1 \log X$ (Spiegel 1961) Thereafter the significance of the regression coefficient a_1 was tested. Further the significance of the results was also tested with the chi-squared test

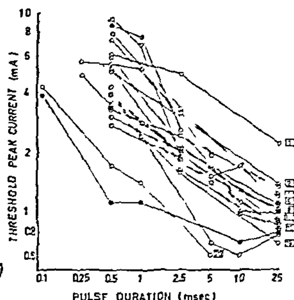


Fig 3 The curves illustrating the relationship between the peak current and the pulse duration at the threshold for the clinical stimulation responses obtained from 20 Parkinsonian patients The curves are plotted on double logarithmic coordinates The open circles denote motor and the filled circles "sensory" responses. The pulse repetition frequency was 1/sec

of patients in whom each of the body areas was involved are plotted against increasing pulse duration. There was a slight tendency towards a decreasing percentage of patients in whom the extremities were involved but the changes were not statistically significant ($P>0.1$) The increasing involvement of the head neck region on the other hand was highly significant ($P<0.001$)

III The peak current pulse duration relationship

Fig 3 shows the curves illustrating the peak current pulse duration relationship at the threshold for the projected clinical responses obtained by electrical stimulation of the Parkinsonian patients Plotted on double logarithmic coordinates the majority of the curves assume a straight line configuration with a definite slope A rheobasic plateau could not be obtained with the pulse durations tested However in some patients (*i e* no 5 10 13 14 17 and 18) the threshold peak current at the longest pulse durations was higher than could have been expected from the first straight part of the curves

The peak current pulse duration curves for the clinical threshold responses of the non-Parkinsonian patients are shown in Fig 4 No significant difference between these and the former curves can be detected

The peak voltage/peak current ratio increased when the length of the pulses applied to brain tissue was increased all the other parameters being kept constant (Johansson 1969) Consequently the peak current pulse duration curve and the peak voltage pulse duration curve had different slopes This is shown in Fig 5 in which both curves obtained from patients no 13 and 20 are illustrated

TABLE II Number of patients in whom the clinical threshold responses, regardless of their character, were projected to one or more of the body regions, the head neck, the upper extremity and the lower extremity The stimulus parameters and the patients are the same as in Table I

Pulse duration (msec)	0.1	0.25	0.5	1	2.5	5	10	25
Total number of patients stimulated	8	8	27	10	22	9	10	26
Number of patients who reacted to stimulation with clinical responses	3	3	26	10	22	9	10	26
Number of patients who reacted at the stimulation threshold with response(s) projected to								
A Head and/or neck	1	—	6	5	15	7	9	23
B Upper extremity	2	3	20	7	18	5	6	18
C Lower extremity	—	1	7	2	6	1	1	4

When the peak current was increased above the threshold values the responses rapidly changed from being more or less localized to the hand, for example, to involving more and more parts of the body This concerned pulses at any duration tested but was most clearly seen with pulses of long durations Fig 6 shows the peak current-pulse duration relationship of muscle groups of different parts of the body (patient no 21, Fig 4) The muscles of the face and upper extremity and the orbicularis oculi muscle seem to have excitability curves of different configuration.

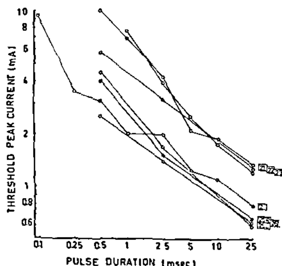


Fig 4 The curves illustrating the relationship between the peak current and the pulse duration at the threshold for the clinical stimulation responses obtained from 5 patients with a hereditary intention tremor and from one patient with an atypical congenital tremor (no 23) Patient no 23 was stimulated *bilaterally* —R denotes the right and L the left brain hemisphere The open circles signify motor and the filled 'sensory responses The pulse repetition frequency was 1/sec

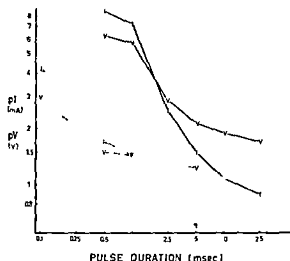


Fig 5

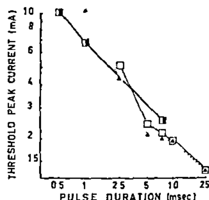


Fig 6

g 5 The peak current—pulse duration (indicated by I) and the peak voltage—pulse di ration (V) curves for the clinical threshold responses obtained from pat ents no 13 (solid lines) and 20 (dashed lines) The curves are plotted on double logarithmic coordinates.

Fig 6 The electrical thresholds needed to excite clinically different muscle groups in pat ent no 21 The black triangles denote the excitation of hand arm muscles the open squares muscles of the face and f nally the half filled squares denote the excitation of the homolateral orbicularis oculi muscle The curves are plotted on double logarithmic coord nates The pulse repetition frequency was 1/sec

Discussion

Some of the stimulations with pulses of 0 1 0 25 and 0 5 msec in duration did not result in any detectable clinical response However this certainly does not mean that the structures stimulated were not excitable with pulses of these durations Rather it indicates that the maximum peak current output (about 10 mA) of the stimulation circuit was not high enough to evoke responses This is in good agreement with the appearance of the peak current pulse duration curves for the threshold responses The slope of the curves indicates that approximately half the number of patients could not respond positively to a stimulation with pulses of a peak current of 10 mA and a pulse duration of 0 1 msec The coordinates of this parameter combination lie in the subliminal area Therefore only the positive responses evoked by pulses of 0 1 to 0 5 msec in duration were taken into consideration for the calculation of the percentages in Fig 1 and 2

In Table I and Fig 1 the responses were divided into motor and sensory responses The character of the motor responses differed slightly regarding stimulus pulse synchronism, strength of the individual twitches local quantitative involvement of muscle units and type of movement with pulses of different durations This was not stressed very much in the results since the recording technique did not permit a reliable systemic recording of such slight differences between the responses

Apart from any effects that may have been produced by the changed pulse duration, this instability of the responses on a more 'local level" was presumably partly due to changes in the alertness of the patient during the experiments and partly to other similar factors that were difficult to control Such an instability was also observed when the target area was stimulated with pulses of constant duration and repetition frequency (Johansson 1969)

The division of the responses according to their localization in the body, as performed in Table II and Fig 2, was quite arbitrary and could have been performed in other ways Thus, for example, the ocular responses could easily have been separated from the others However, such subgroupings would not have changed the main features of the results

In the present study the projected localization of the threshold responses was correlated with the pulse duration. However, as soon as the pulse duration was changed from one value to another the peak current also had to be changed in order to maintain a threshold stimulation When the pulse duration was increased, the peak current had to be decreased and vice versa An increasing peak current is known to change the clinical responses from single responses to more complex ones, i e probably to increase the volume of the affected neuronal pool (*cf e g* Fritsch and Hitzig 1870, Bosma and Gellhorn 1946, Liddell and Phillips 1951 Cure and Rasmussen 1954 and Gurevitch and Abeles 1967) Conversely, a decreasing peak current will decrease the affected pool A decrease of the excited neuronal pool can hardly be supposed to result in increasing involvement of the head muscles, such as was shown in the present study Therefore the increased pulse duration may very probably have been responsible for the phenomenon Further, the changing peak voltage/peak current ratio resulting from the change of the pulse duration may, though not very probably, be of importance

It is interesting to note that Wyss and Obrador (1937) found slower optimum currents for proximal than for distal movements when they stimulated the motor cortex of monkeys with single stimuli delivered from a condenser discharge stimulator

Cure and Rasmussen (1954) found on stimulating the precentral gyrus of the brain of *Macaca mulatta* with pulses ranging from 0 1 to 3 msec in duration, that longer pulses occasionally favoured movements involving more proximal muscles than the shorter pulses did Mihailovic and Delgado (1956) found on stimulating the monkey brain, that with longer pulse durations at threshold stimulation a greater number of muscle units were simultaneously involved in the responses than when pulses of shorter duration were applied

Glusman *et al* (1952 and 1953), on the other hand in studies on the effects of electrical stimulation of the human motor cortex and uncus with pulses of different durations (0 1 to 5 msec), obtained results which are not in accord with those of the present study They found only minor changes, if any in the responses with different pulse durations

Landgren, Phillips and Porter (1962) applied surface-anodal current pulses to

the origin of the corticospinal tract in baboons and simultaneously performed microelectrode recordings from the lateral corticospinal tract and alpha motoneurones of the hand and forearm Brief (0 2 msec) pulses were shown to send single volleys down the contralateral tract With repetitive stimulation the successive monosynaptic excitatory post synaptic potentials increased in amplitude *i e* the transmitting action of pyramidal synapses improved with repetition Long (5 msec) pulses caused high frequency discharge of the corticofugal cells and the resulting increasing monosynaptic action of these pulses was concluded to account for their special efficacy in evoking movements

It seems reasonable to suppose that the different neuronal trajectories threshold stimulation of which is able to evoke clinical responses differing in projected localization react at different combinations of peak current and pulse duration This was in fact indicated by the results shown in Fig 6 where it is seen that at least for the patient in question (no 21) the pathways conveying impulses to the hand face and orbicularis oculi muscles needed a peak current of a different intensity at a given pulse duration in order to excite the muscles clinically *A similar phenomenon as noticed in the responses of several patients but shortage of time did not permit* recording of supraliminal responses in the present study Liddell and Phillips (1950 and 1951) came to similar conclusions when they stimulated the motor cortex of the baboon with pulses of a constant duration (5 msec) They stated The threshold is always lowest for the thumb complex usually higher for the toe complex and highest for the face complex

The peak current pulse duration curves shown in the present study cannot very easily be compared to other strength-duration curves for clinical threshold responses obtained by stimulation of the central nervous system (*cf e g* Glusman *et al* 1952 and 1953 Mihailovic and Delgado 1956 Libet *et al* 1964 and Sugita and Doi 1967) The most important reasons for this are probably the following facts Firstly an electrical threshold for a given response obtained with a constant current" stimulation is difficult to compare with the threshold for a similar response obtained with a constant voltage stimulation (*e g* Lale 1965) Most of the earlier curves were obtained with a constant voltage stimulation Secondly the projected localization of the responses in the present study was different in the d fferent parts of the curves This indicated the involvement of many different neuronal trajectories rather than only one The present curves thus show the peak current at given pulse durations below which not one of the family of neuronal trajectories will be stimulated enough to result in clinical responses Thirdly peak current pulse duration curves for clinical threshold responses obtained by stimulation of the human ventrolateral and subventrolateral thalamic structures have not as far as I know been presented earlier

Conclusions

Electrical square wave pulses with a constant repetition frequency of 1 pulse/sec and durations ranging from 0 1 to 25 msec were applied to a human ventrolateral

subventrolateral thalamic target area. In the majority of the patients tested the stimulation resulted in clinical threshold responses consisting of muscular jerks or twitches of different contralateral muscles or muscle groups

When the pulse duration was increased, the peak current had to be correspondingly decreased in order to maintain a threshold stimulation. In the majority of the patients, the relationship between the two parameters, plotted on double logarithmic coordinates, assumed a straight line configuration within the ranges of the parameters tested

The character of the threshold responses remained the same for all pulse durations tested

Short pulses (<1 msec) resulted in threshold responses mainly projected to the upper extremity but in some cases also/only to the lower extremity and/or the head With an increasing pulse duration the head region was involved in the response in an increasing number of patients

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From the Neurosurgical Clinic, Helsinki, Finland

Electrical Stimulation of a Human Ventrolateral-Subventrolateral Thalamic Target Area

III. Combination of different pulse repetition frequencies with the peak current just adequate to evoke clinical responses

By

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Abstract

JOHANSSON, G G *Electrical stimulation of a human ventrolateral-subventrolateral thalamic target area III Combination of different pulse repetition frequencies with the peak current just adequate to evoke clinical responses* Acta physiol scand 1969 *75* 456—467

A ventrolateral-subventrolateral thalamic target area was electrically stimulated in connection with stereotaxic neurosurgery in 16 alert patients with Parkinson's disease, in 4 patients with hereditary intention tremor and in 1 patient with choreoathetosis The stimulations were performed as a part of a larger investigation concerned with the effect of altering stimulus parameters on the clinical responses evoked Unidirectional square wave pulses with a constant pulse duration and repetition frequencies ranging from 1 to 200 pulses per second were applied The stimulation was kept at the threshold for clinical responses by balancing the different repetition frequencies with the peak current The stimulation elicited motor and "sensory" responses The character of the responses depended on the pulse repetition frequency used The projected localization of the responses in the body remained unchanged The peak current pulse repetition frequency relationship at the threshold for evoking clinical responses is shown The results are discussed

The application of electrical pulses to the motor cortex of the monkey has been shown to elicit movements with a projected localization depending on the pulse repetition frequency used (e g Liddell and Phillips 1950 and 1951 Cure and Rasmussen 1954 and Mihailović and Delgado 1956) In a previous study, the projected localization of the clinical motor and "sensory" threshold responses, obtained by the application of single electrical pulses to a human ventrolateral subventrolateral thalamic area, was also shown to depend on the duration of the pulses (Johansson 1969 b)

The present study is concerned with the clinical threshold responses evoked by electrical stimulation of the same subcortical area with pulses of different repeti-

tion frequencies The duration of the pulses was kept constant for each patient, in order not to mix the effects resulting from changing it with the effects of the altered pulse repetition frequency In addition, the peak current pulse repetition frequency relationship at the threshold for clinical responses is studied and discussed

Methods

son 1969 a) and 1969 b) uated in the The metric of the third ventricle and 16 0—17 0 mm posterior to the anterior commissure The very tip of the monopolar electrode lay on the intercommissural line The unidirectional square wave pulses were applied through an electrode which had an uninsulated blunt tip of 18 mm² The "active electrode was cathodal with respect to an indifferent remote electrode A stimulator with a constant current" output was used The current through and the voltage drop across the patient were monitored and recorded

Threshold stimulations with pulse repetition frequencies ranging from 1 to 200 sec were carried out The train repetition frequency was kept at 1 train per minute or slower The train duration varied between 5 and 10 sec The pulse duration was kept constant at a value individual for each patient (= "chronaxy') How this was determined will be described later on. The peak current was continuously changed during every stimulus train in order to find the stimulus threshold According to the stimulation technique used (Johansson 1969 a), it was increased during the first train until clinical responses were observed During the next stimulation the current was decreased from the supraliminal value just achieved until the last response had vanished This response was recorded regardless of whether it was sensory ' or motor in character or of its projected localization The peak current just able to elicit it was called the threshold peak current

Alternative patients were stimulated starting from the high and the others starting from the low pulse repetition frequencies Every patient was stimulated with at least 3 different pulse repetition frequencies representing the ranges 1 5—20 and 50—200 pulses/sec

The constant pulse duration

The pulse duration to be kept constant was chosen individually in the following way A h a pulse duration was increased and found in this way he following stimulation the was recorded In performing th supraliminal) towards a shorter p way called chronaxy for the clir and 6 2 msec (mean and standard deviation 3 5 ± 1 0 msec

Patients

Twenty-one patients were stimulated Sixteen of them had Parkinson s disease 4 had a hereditary intention tremor and 1 had choreoathetosis One of the 4 patients with hereditary intention tremor was a 24 year-old woman and the one with choreoathetosis was a 26 year-old man The age of the other patients varied between 46 and 67 years the mean age being 57 (SD ± 6) years There were 11 men and 10 women Two of the patients had been treated stereotaxically in the other brain hemisphere earlier and 1 in the same hemisphere but in a slightly different operation target area 16 of the operations were performed in the left and 5 in the right hemisphere

Results

All stimulation responses described in the text and in the tables were contralateral to the side of the brain stimulated The tremor "increase' and ' decrease , respectively denote changes of tremor amplitude, as visually estimated

I The effect of different pulse repetition frequencies on Parkinsonian tremor

In most cases, insertion of the electrode into the target area stopped the Parkinsonian tremor either immediately or within a few minutes of insertion of the electrode For the minority of the patients in whom the tremor persisted, stimulations were applied during quiet periods This was important because of the difficulty of interpreting the effect of stimulation when a simultaneous tremor was present

However, if a tremor still persisted during stimulation, pulses applied once a second mostly modulated it, *i e*, the tremor was superposed by muscular jerks corresponding to the stimuli Pulse repetition frequencies of 5, 10, and 20/sec increased a weak or moderate tremor With a 50 pulses/sec stimulation the effects were variable, the tremor either increased, decreased or remained unchanged Sometimes, after an initial stop, it was shown to start again if the train duration was long enough and especially if the peak current was kept at supraliminal values If tremor was present on stimulation with pulse repetition frequencies of 100 and *200/sec it was usually damped*

II Character of the responses (no initial tremor)

One pulse per second The threshold responses for this pulse repetition frequency were stimulus synchronous twitches or jerks of the orbicularis oris muscle and of various hand and arm muscles In some cases twitches occurred in orbicularis oculi or leg muscles

Five pulses per second The only type of response evoked with this repetition frequency was tremor of the lips and hand The tremor seemed to be stimulus synchronous

Ten pulses per second Besides a tremor evoked with this repetition frequency some of the patients reported sensations of tingling or electricity located to the face or extremities Sometimes the sensations were combined sometimes not with a simultaneous visible tremor

Twenty pulses per second This repetition frequency also produced tremor which was usually irregular with a slow frequency but having a clearly larger amplitude than the tremor evoked by 5 and 10 pulses/sec Sensations of electricity or of tingling were *reported more frequently than with 10 pulses/sec*

The tremor resulting from the application of pulses of repetition frequencies of 5, 10, and 20 pulses/sec mostly started at a definite point when the peak current was steadily increased from a low to a higher intensity However it almost always outlasted the stimulation by 5 to 10 sec after the peak current had been decreased below the starting threshold and likewise if stimulation was totally interrupted Therefore, the exact decreasing current thresholds were sometimes rather difficult to determine with these pulse repetition frequencies In some cases they had to be approximated

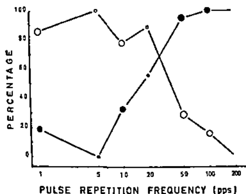


Fig 1

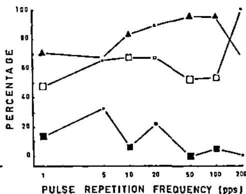


Fig 2

Fig 1 Percentages of patients from Table I presenting "sensory" (filled circles) and/or motor (open circles) threshold responses The size of the circles indicates the relative number of patients stimulated at a given repetition frequency The pulse repetition frequency axis is logarithmic The changes in the character of the responses are highly significant ($P<0\ 001$)

Fig 2 Percentages of patients from Table II presenting threshold responses projected to different parts of the contralateral side of the body The symbols denote percentages of patients with responses situated in the upper extremity (triangles), the head neck region (open squares) and the lower extremity (filled squares) The size of the symbols indicates the relative number of patients stimulated with a given pulse repetition frequency The variations of the percentages at different repetition frequencies are not significant ($P>0\ 1$)

Fifty, a hundred and two hundred pulses per second The threshold responses evoked in the majority of the patients with these repetition frequencies were sensations of tingling or of electricity The patients had difficulty in explaining what they really experienced They had never experienced anything similar before Some of the patients said that the sensation was like a movement of electricity When the extremities in which these sensations were present were passively moved by the observers during stimulation, no definite changes of the muscle tone could be detected At the thresholds or at slightly supraliminal current values the sensations were not uncomfortable but at higher values they became unpleasant without resembling any clear cut pain quality

In 1 patient (no 6, Fig 3) the sensations experienced differed from those usually reported in this series Pulses applied once every second resulted in a sensation of pulsation in the wrist With higher pulse repetition frequencies the sensation evoked became burning in character Further the patient experienced a feeling of coldness in her throat in the initial part of the stimulus train which after a few seconds changed to warmth

In the small minority of the patients in whom the tremor had persisted the motor responses at the stimulation threshold with repetition frequencies of 50, 100 and 200 pulses/sec consisted of changes in this initial tremor Further, in a relatively poorly cooperating 61-year old woman (patient no 1, Fig 3) 100 pulses/sec resulted in flexion of the elbow and deviation of both eyes in a lateral direction away from the

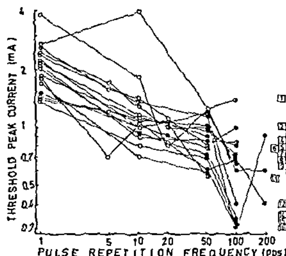


Fig 3 The curves showing the relationship between the peak current and the pulse repetition frequency at the threshold for clinical responses obtained in 16 Parkinsonian patients The curves are plotted on double logarithmic coordinates The pulse duration was kept constant = "chronaxy" The open circles denote motor and the filled circles "sensory" responses

stimulating electrode The same repetition frequency caused muscular tension in the lower lip of patient no 4 (Fig 3) and 50 and 100 pulses/sec resulted in thumb extension and grimacing of the face of the patient with choreoathetosis (no 18, Fig 4) It is worth mentioning that in a further 2 patients (no 3 and 20, Fig 3 and 4, respectively) a very slightly supraliminal peak current resulted in extension of the fingers and muscular tension of the hand and arm, respectively The pulse repetition frequency at which this happened was for the former patient 100 and for the latter 50/sec

The threshold responses were classified as either motor or "sensory" in character (Johansson 1969 a) Every kind of definite clinical muscle excitation evoked by the stimulation was counted as a motor response "Sensory" responses were those sub

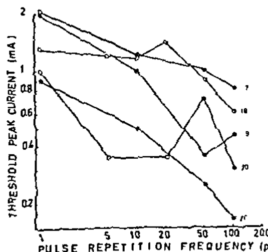


Fig 4 The curves showing the relationship between the peak current and the pulse repetition frequency at the threshold for clinical responses obtained from 4 patients with a hereditary intention tremor and from one patient with choreoathetosis (no 18) Pulse duration and symbols as in Fig 3

TABLE I Number of patients presenting motor and/or "sensory" threshold responses on stimulation of the ventrolateral subventrolateral thalamic target area The pulse repetition frequency varied from 1 to 200/sec and the corresponding peak current from 4 to 0 2 mA The pulse duration was kept constant = "chronaxy"

Pulse repetition frequency (pulses per second)	1	5	10	20	50	100	200
Total number of patients stimulated	21	6	18	9	21	19	3
Number of patients who reported "sensory" responses at the stimulation threshold	4	—	6	5	20	19	3
Number of patients who reacted with motor responses at the stimulation threshold	18	6	14	8	6	3	—

jectively experienced by the patient during stimulation But sensations of movement, jerks or tremor when corresponding motor activity was visually present were not counted as "sensory" responses Occasionally, some of the patients had sensations of jerks or tremor without simultaneously visible corresponding muscular activity These sensations were recorded as "sensory" responses The pulse repetition frequencies at which "sensory" responses of this quality were obtained were 1 5, 10 and 20/sec

Table I shows the number of patients stimulated in whom the projected threshold responses were motor "sensory" or both simultaneously in character In Fig 1 it is shown that the percentage of the patients from Table I presenting motor responses diminished when the pulse repetition frequency was increased The percentage of patients presenting "sensory" responses, on the other hand, rose In either case the changes were highly significant ($P<0\ 001$) For the statistical methods used see Johansson (1969 b)

III Projected localization of the responses

Table II shows the number of patients in whom the threshold responses evoked were projected to 1 or more of the three parts of the body 1) the head and neck, 2) the upper extremity and 3) the lower extremity For each repetition frequency every patient could maximally contribute once to every one of the regions regardless of whether the response involving the area was motor or "sensory" in character or both simultaneously In Fig 2 the percentages of patients in whom each of the regions was involved are plotted against the pulse repetition frequency The small changes are not significant ($P>0\ 1$)

TABLE II. Number of patients in whom the clinical threshold responses, regardless of their character, were projected to one or more of the body regions head-neck, upper extremity and lower extremity The stimulus parameters and the patients are the same as in Table I

Pulse repetition frequency (pulses per second)	1	5	10	20	50	100	200
Total number of patients stimulated	21	6	18	9	21	19	3
Number of patients who reacted at the stimulation threshold with response(s) projected to							
A Head and/or neck	10	4	12	6	11	10	3
B Upper extremity	15	4	15	8	20	18	2
C Lower extremity	3	2	1	2	—	1	—

IV The peak current—pulse repetition frequency relationship

The curves showing the relationship between the peak current and the pulse repetition frequency at the threshold for clinical responses obtained by stimulation of the 16 Parkinsonian patients are illustrated in Fig 3 Plotted on double logarithmic coordinates, the majority of the curves assume a straight-line configuration between 1 and 50 pulses/sec The threshold peak current at 100 pulses/sec was significantly lower than was to be expected from the earlier part of the curves

The corresponding curves obtained by stimulation of the non-Parkinsonian patients are illustrated in Fig 4

Discussion

As far as possible, stimulation was applied when all signs of Parkinsonian tremor were absent, in order to obtain a uniform basis for the experiments In the great majority of the patients this was possible, since, as earlier described, the mere insertion of the electrode into the target area usually stopped the tremor In some of the patients, however, moderate or slight tremor persisted Further, a few patients showed occasional tremor bursts between and during the experiments Therefore, some of the stimulations had to be performed in spite of tremor influencing the recordings and making them less reliable

Although the minority of patients in whom a slight tremor was present during the stimulations were not quite comparable with those without a tremor, all the patients stimulated, without exception, were included in the material This was done because it seemed difficult to draw a distinction between the patients on grounds of such slight and often temporary differences in tremor status

It is worth mentioning that the effect on a Parkinsonian tremor of electrical stimulation applied to the present target area was essentially similar to that reported

by Hassler and Riechert (1961) to occur on stimulation of the ventro-oral nuclei of the thalamus

The "chronaxy" was chosen as constant pulse duration in an attempt to smooth out individual differences in excitability It was thought that the curves showing the peak current pulse repetition frequency relationship in this way would be easier to compare with each other Whether this succeeded or not is impossible to conclude—the individual variations of the curves were still considerable The parts of the curves obtained in the range between 1 and 50 pulses/sec grossly resemble those illustrated by Libet *et al* (1964), who stimulated the human somatosensory cortex Almost similar peak current pulse repetition frequency relationships were also illustrated in animal experiments by Lilly, Austin and Chambers (1952) Their curves were obtained by stimulation of the cerebral motor cortex of cats and monkeys

Between the later part of the present curves and of those shown by the above-mentioned investigators, however, there was some discrepancy The slope of their curves was more or less straight with all pulse repetition frequencies up to 100 or more per second In the present study, on the other hand, there was a significant drop of the threshold peak current between 50 and 100 pulses/sec The definite change of the threshold responses from motor to "sensory" at 50 pulses/sec is probably of significance in this respect

Responses evoked by electrical stimulation of many different brain areas, both in man and in animals have been shown to be dependent on the pulse repetition frequency (*cf e g* Hines 1940 Hare and Geohegan 1941, Berry McKinley and Hodes 1942, Liddell and Phillips 1950 and 1951, Moruzzi 1950, Hyde and Gellhorn 1951, Kaada 1951, Lilly Austin and Chambers 1952 Cure and Rasmussen 1954, Mihailovic and Delgado 1956, Buchwald *et al* 1961, Gengerelli 1961, Libet *et al* 1964, Bergstrom *et al* 1966 and Traczyk and Jakubowska 1966)

It is also well known that single stimuli applied to certain human thalamic structures result in muscular twitches and that stimuli of higher repetition frequencies often cause subjective sensations (*cf e g* Ervin and Mark 1960, Hassler and Riechert 1961 and Schaltenbrand 1965)

Kaada (1951) suggested two possible explanations of the dependence of the stimulation responses on the pulse repetition frequency Firstly, two groups of neurones subserving two different functions could be excited selectively Secondly the same group could convey impulses into different synaptic channels according to the different pulse repetition frequencies

Lilly Austin and Chambers (1952) who studied the cortex of monkeys by electrical stimulation concluded that short pulses at low repetition frequencies (less than 5 to 10/sec) excited only efferent fibres while at higher frequencies mainly cortical strata were being stimulated

Further, it has been shown that stimulation of a complex synaptic network (motor cortex of cats and monkeys) may lead to direct excitation of the outflow neurones and to excitation of interneurones in the network (Amassian and Patton

1961) At high pulse repetition frequencies the interneuronal action on the outflow discharge was largely absent

The present peak current pulse repetition frequency curves with their straight line configuration between 1 and 50 and the following drop of the threshold between 50 and 100 pulses/sec are interesting in the light of the suggestions and experimental results mentioned In the majority of the patients the projected threshold responses at pulse repetition frequencies from 1 to 20/sec were motor With 50 to 200 pulses/sec, on the other hand the threshold responses were in most cases 'sensory' in character We also know that stimulation of the same target area during general anesthesia with 60 pulses/sec, in cerebral palsy patients at least, resulted in tonic contractions of hand, arm and leg muscles, deviation of the eyeballs and changes of the respiratory rate (Bergstrom *et al* 1966) The peak current needed to evoke these responses was very much higher than that necessary, at a corresponding pulse repetition frequency to evoke the 'sensory' responses reported in the present study This, of course, was partly due to the threshold increasing effect of the general anesthesia used but might further partly have been due to a true higher threshold for motor responses at this pulse repetition frequency than for "sensory" responses In the present stimulation series on alert patients it was not possible to test for motor responses at high pulse repetition frequencies by increasing the peak current since the low threshold sensory responses became unpleasant for the patient at supraliminal current values However, as described in the results 3 of the patients at the stimulation threshold and 2 at slightly supraliminal peak current values presented clinically increased muscle tension or muscular contractions when pulses of a repetition frequency of 50 and 100 pulses/sec were applied Therefore it seems quite possible, on the basis of these facts and the configuration of the peak current pulse repetition frequency curves shown, that the motor at low and the sensory threshold responses at high pulse repetition frequencies have the lowest current thresholds Thus the curves indicating the thresholds for motor and sensory responses respectively would follow different courses and intersect each other at approximately the repetition frequency of 50 pulses/sec

According to the stereotaxic atlas of Schaltenbrand and Bailey (1959) the internal capsule passed only 1 to 2 mm lateral to the tip of the electrode This may easily lead one to believe that the motor effects were due to the direct excitation of motor efferents This was also concluded by Albe Fessard *et al* (1966) who measured the latency of the muscle responses evoked and found the time interval to be similar in length whether the capsular or thalamic structures were being stimulated However, this conclusion seems to be at variance with the findings of Bertrand Blundell and Musella (1965) for example These workers from the results of direct stimulation of the internal capsule in man concluded that at the level of the intercommissural line the corticobulbar and corticospinal fibres were situated behind the midpoint of this line, 17 to 22 mm from the midline This means that the motor efferents pass 5 to 10 mm lateral to the present stimulation target area

Hassler and Riechert (1961) believed that the muscular twitches evoked by low

repetition frequency stimulation of the ventro-oral nuclei of the thalamus are due to excitation of thalamo-cortico-spinal trajectories

The origin of the "sensory" responses is uncertain. The sensations evoked by pulse repetition frequencies above about 20/sec seem to be very similar to those which can be evoked by stimulation of the human sensory cortex (*cf* *e g* Penfield and Boldrey 1937 and Libet *et al* 1964) It is interesting to note that the responses evoked by stimulation of the sensory cortex resemble those reported in the present study in other respects as well Libet *et al* (1964) applied square wave pulses of many different parameter combinations and observed that single pulses elicited muscular twitches, whereas pulses applied with repetition frequencies of 15/sec or up, with a train duration of 1 to 10 sec, resulted in sensations only Sensations of the type reported in the present study evoked from human thalamic-subthalamic structures have been reported and discussed by several authors (*e g* Alberts *et al* 1961 and 1966 Bertrand, Blundell and Musella 1965, Johansson and Laitinen 1965 and Andy 1966) Johansson and Laitinen (1965) and Andy (1966) have suggested that the sensation may be due to excitation of the proprioceptive elements However, no evidence to support this hypothesis is available The sensations of pulsations and of heat, atypical of this series of stimulations, described by 1 of the patients, resembled the sensations described by patients stimulated in the medial lemniscus just below the nucleus limitans portae of the thalamus (Johansson 1968)

Conclusions

Electrical square wave pulses with a constant pulse duration and repetition frequencies ranging from 1 to 200 pulses/sec were applied to a human ventrolateral subventrolateral thalamic target area The clinical responses evoked by pulses of the lowest effective peak current were recorded They consisted of motor and sensory responses projected to the head and the extremities of the contralateral side of the body

When the pulse repetition frequency was increased the peak current had to be correspondingly decreased in order to maintain a threshold stimulation Plotted on double logarithmic coordinates the curves showing the relationship between the two parameters had an almost straight line configuration between 1 and 50 pulses/sec With a pulse repetition frequency of 100/sec the threshold peak current was lower than could have been expected from the straight line slope between 1 and 50 pulses/sec

The character of the threshold responses depended on the pulse repetition frequency used One and 5 pulses/sec evoked muscular twitches only 10 and 20/sec resulted in tremor but also in sensations of electricity or of tingling In the majority of the patients higher repetition frequencies at the stimulation threshold resulted in "sensory" responses only

The projected localization of the responses in the body remained the same for all pulse repetition frequencies tested

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Electrical Stimulation of a Human Ventrolateral-Subventrolateral Thalamic Target Area

IV. Combination of different pulse repetition frequencies with the pulse duration just adequate to evoke clinical responses

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Abstract

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A ventrolateral subventrolateral thalamic target area was electrically stimulated in connection with stereotaxic neurosurgery in 20 alert Parkinsonian patients and in 4 patients with hereditary intention tremor The study forms part of a larger investigation concerned with the effect of different stimulus parameters on the clinical responses evoked by stimulation Here unidirectional square wave pulses with a constant peak current and repetition frequencies ranging from 1 to 200 pulses per second were applied The stimulation was kept at the threshold for clinical responses by balancing the different repetition frequencies with the pulse duration Stimulation elicited motor and "sensory' responses The character of the responses depended on the pulse repetition frequency used The localization in the body of the projected threshold responses, on the other hand depended on the pulse duration used The pulse duration pulse repetition frequency relationship at the threshold for evoking clinical responses is shown The results are discussed

In a previous study (Johansson 1969 b) single electrical square wave pulses were applied to a human ventrolateral-subventrolateral thalamic target area The area of *localization in the body of the projected clinical threshold responses increased* when the duration of the pulses was increased

The application of pulses of different repetition frequencies (1 to 200 pulses/sec) but with a constant pulse duration, evoked responses the projected localization of which remained unchanged at all frequencies tested (Johansson 1969 c) The character of the responses, however, was different—with low repetition frequencies it was mainly motor and with frequencies of over 20 pulses/sec sensory in type

It was to be expected that both the projected localization and the character of

the threshold responses would change if both the duration and the repetition frequency of the pulses were changed simultaneously In the present study the results of such stimulations applied to the same target area as in the above mentioned studies are shown. Experiments were performed on 24 patients in connection with stereotaxic neurosurgery The relationship between the two parameters at the threshold for clinical responses has also been studied and is discussed

Methods

The surgical and stimulation methods were described in detail previously (Johansson 1969 a) The stimulation target area was the same as in the earlier studies (Johansson 1969 a, b and c) It was presumably situated in the ventro-oral nuclei (nomenclature in accordance with Schaltenbrand and Bailey 1959) of the thalamus at the boundary between there and the subthalamic structures The very tip of the monopolar stimulating electrode lay on the inter commissural line 11.5—13 0 mm lateral to the midline of the third ventricle and 16 0—17 0 mm posterior to the anterior commissure The unidirectional square wave stimuli were applied through a porcelain insulated steel electrode which had an uninsulated blunt tip 2 8 mm in length and 2 mm thick The electrode was cathodal with respect to an indifferent remote electrode The current through and the voltage drop across the patient were monitored A stimulator with a constant current output was used

Threshold stimulations were carried out by applying pulses with different repetition frequencies The duration of the stimulus trains varied between 5 and 10 seconds The intervals between the different stimulus trains were at least 1 minute Every patient was stimulated with at least 3 different pulse repetition frequencies representing the ranges 1 5—20 and 50—200 pulses/sec Alternate patients were stimulated starting with high repetition frequencies and the others starting with low frequencies The peak current was kept constant at a value called the rheobase for clinical responses This was experimentally determined in every patient in a way which will be described in detail later on.

According to the stimulation technique used (Johansson 1969 a) the pulse duration was increased during the first stimulus train applied until clinical responses were observed During the next stimulus train it was again decreased from the previously achieved slightly supraliminal value Now the clinical threshold responses were recorded regardless of whether they were sensory or motor in character or both and of their projected localization in the body The shortest pulse duration adequate to evoke threshold responses was simultaneously recorded It was called the threshold pulse duration

The constant peak current

The peak current to be kept constant was chosen individually in the following way A stimulation was carried out with pulses applied once every second The duration of these pulses was 25 msec During the first stimulation the peak current was increased until clinical responses were observed Thereafter a second stimulus train was applied and the peak current was decreased from the supraliminal value just achieved until clinical responses could no longer be observed The least peak current adequate to evoke clinical responses was regarded as the rheobase The value of this rheobase varied in the different patients between 0 4 and 2 mA (mean and standard deviation 1 2 ± 0 4 mA

Patients

T
axic neurosurgery Twenty of
tremor One of the patients
and 69 years and the mean
14 of the stimulation series were carried out in the left and 10 in the right brain hemisphere Four of the patients had previously undergone stereotaxic brain operations 3 in the opposite hemisphere and 1 in the same hemisphere but in a slightly different target area

Results

The stimulation responses were contralateral to the side of the brain stimulated Tremor "increase" and "decrease" respectively denote changes of tremor amplitude as visually estimated

I Character of the responses

In their general character the responses were similar to those evoked by pulses of different repetition frequencies but with the pulse duration kept constant (Johansson 1969 c), instead of the peak current, as in the present study Therefore, only a summary of the character of the responses is given here

Pulses applied once every second resulted in twitches of different muscles or muscle groups of the head and extremities When an initial tremor was present, it was modulated by these twitches Five pulses/sec resulted in a stimulus-synchronous tremor in lips or/and extremities A tremor was also caused by pulses applied at a *rate of 10 and 20/sec This tremor* was irregular, with a large amplitude *At these* frequencies, independently of or simultaneously with the tremor, some of the patients had sensations of tingling or of electricity in the face and/or extremities 50 and 60 pulses/sec resulted in similar subjective sensations but only seldom in motor responses If a tremor was present when a stimulation with these repetition frequencies was performed, the effect was variable The tremor either increased, decreased or remained unchanged Higher pulse repetition frequencies caused subjective sensations in the great majority of the patients and if a tremor was present it was usually damped by stimulation

Responses diverging from the general ones were recorded in 4 patients Patient no 1 (Fig 3) reported a feeling of discomfort in her laryngeal tract when pulses were applied at a repetition frequency of 60/sec The threshold response evoked with a stimulation of 50 and 200 pulses/sec in a 69 year-old woman (no 4 Fig 3) was increased tone of the muscles of the lower jaw This woman, however, cooperated rather poorly regarding subjective sensations The insertion of the electrode into the target area of a 50 year-old man (no 5, Fig 3) resulted in a persistent spontaneous Babinski sign The stimulation of this patient with pulses applied once every sec resulted in bilateral nystagmus At higher pulse repetition frequencies the patient reported sensations of warmth or heat in the hand A 63-year-old woman with hereditary intention tremor (patient no 23 Fig 4) reported a slightly uncomfortable sensation of humming in her contralateral temporal region The pulse repetition frequencies evoking this sensation were 100 and 200/sec

The character of all the threshold responses was classified as either motor or "sensory" in type All kinds of definite clinical changes in muscular activity evoked by the stimulation were regarded as motor responses while those subjectively experienced by the patients were recorded as "sensory" responses Sensations of muscular twitches or tremor in parts of the body in which corresponding motor activity *was simultaneously evoked visually were not recorded as* "sensory" responses

TABLE I Numbers of patients presenting motor or/and "sensory" threshold responses evoked by stimulation of the ventrolateral subventrolateral thalamic target area The pulse repetition frequency varied from 1 to 200/sec and the corresponding mean threshold pulse duration from 20 5 to 0 18 msec The peak current was kept constant = "rheobase"

Pulse repetition frequency (pulses per second)	1	5	10	20	50—60	100	200
Mean pulse duration at the stimulation threshold (msec)	20 5	7 9	5 1	3	1 6	0 22	0 18
Total number of patients stimulated	24	7	16	13	24	8	17
Number of patients who reported 'sensory' responses at the stimulation threshold	1	—	8	12	22	8	16
Number of patients who reacted with motor responses at the stimulation threshold	24	7	10	10	5	1	4

Table I shows the number of patients in whom stimulation with different repetition frequencies evoked motor and/or "sensory" threshold responses regardless of their projected localization in the body Fig 1 shows the number of the patients from Table I presenting motor and/or 'sensory" responses, respectively, as percentages of all patients stimulated The percentages are correlated to the increasing pulse repetition frequency The decrease in the percentage of patients with motor responses and the corresponding increase of those with 'sensory' responses were both highly significant ($P<0\ 001$)[1]

II Projected localization of the responses

Table II shows the number of patients in whom the responses evoked were projected to either one or more of the three parts of the body 1) the head and neck 2) the upper extremity and 3) the lower extremity The higher the pulse repetition frequency, the shorter was the threshold pulse duration(Fig 3 and 4) The mean values of this, corresponding to the different repetition frequencies are also shown in Table II In Fig 2 the percentage of patients in whom each of the above mentioned body regions was involved in the responses is plotted against the mean values of the threshold pulse duration The percentage in whom the head-neck region was involved is seen to increase with increasing pulse duration ($P<0\ 01$) Tested with the chi squared test the change is only almost significant ($P<0\ 05$) The involvement of the other two body regions also changed slightly at different pulse durations However, these changes are not significant ($P>0\ 1$)

[1] The statistical analysis was performed by solving the regression equation $Y = a_0 + a_1 \log X$ (Spiegel 1961) Thereafter, the significance of the regression coefficient a_1 was tested The significance of the results was also tested with the chi squared test

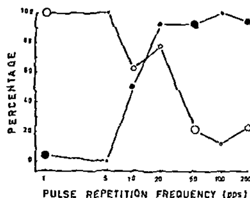


Fig 1

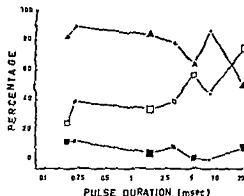


Fig 2

Fig 1 Percentages of patients from Table I presenting "sensory" (filled circles) and or motor (open circles) threshold responses at different pulse repetition frequencies The size of the circles indicates the relative number of patients stimulated at a given repetition frequency The pulse repetition frequency axis is logarithmic The changes in the character of the responses are highly significant ($P<0\,001$)

Fig 2 Percentages of patients from Table II presenting threshold responses projected to different parts of the contralateral side of the body The percentages are correlated to the mean threshold pulse durations The symbols denote percentages of patients with responses situated in the upper extremity (triangles), the head neck region (open squares), and the lower extremity (filled squares) The size of the symbols indicates the relative number of patients stimulated with a given pulse repetition frequency The percentage of patients with responses situated in the head neck region increased with increasing mean threshold pulse duration ($P<0\,01$)

TABLE II Number of patients presenting threshold responses, regardless of type, projected to the head neck, upper extremity and/or the lower extremity evoked by stimulation of the ventrolateral subventrolateral thalamic target area The stimulus parameters were the same as for Table I

Pulse repetition frequency (pulses per second)	1	5	10	20	50—60	100	200
Mean pulse duration at the stimulation threshold (msec)	20 5	7 9	5 1	3	1 6	0 22	0 18
Total number of patients stimulated	24	7	16	13	24	8	1
Number of patients who reacted at the stimulation threshold with response(s) projected to							
A Head and/or neck	18	3	9	5	8	3	4
B Upper extremity	12	6	10	10	20	7	14
C Lower extremity	2	—	—	1	1	1	2

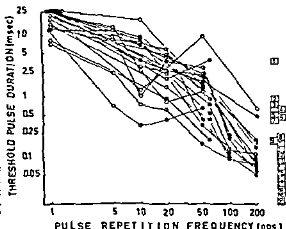


Fig 3 The curves showing the relationship between the pulse duration and the pulse repetition frequency at the threshold for clinical responses obtained in the 20 Parkinsonian patients The curves are plotted on double logarithmic coordinates The peak current was kept constant = 'rheobase' Open circles denote motor and filled circles "sensory" responses

III The pulse duration pulse repetition frequency relationship

The pulse duration pulse repetition frequency relationship at the threshold for clinical responses obtained by stimulation of the 20 Parkinsonian patients is illustrated in Fig 3 In the range between 1 and 50 pulses/sec the slope of most of the curves had an almost straight line configuration if plotted on double logarithmic coordinates In the majority of the patients the threshold pulse duration was shorter at 100 and 200 pulses/sec than could have been expected from the inclination of the first part of the curves

The corresponding pulse duration pulse repetition frequency relationship obtained by stimulation of the 4 patients with a hereditary intention tremor is illustrated in Fig 4

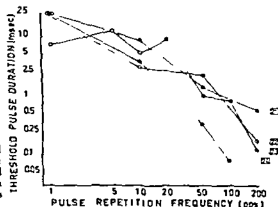


Fig 4 The curves showing the relationship between pulse duration and pulse repetition frequency at the threshold for clinical responses obtained in 4 patients with hereditary intention tremor Peak current and symbols as in Fig 3

Discussion

The curves showing the relationship between the threshold pulse duration and the pulse repetition frequency were very similar to those showing the relationship between the threshold peak current and the pulse repetition frequency previously studied (Johansson 1969 c) Likewise, changes in the type of response along the curves were similar However, the duration of the pulses influenced the projected localization of the threshold responses in a way not observed for the peak current (Johansson 1969 c) The responses, which with short pulses were projected to the extremities in the majority of the patients, involved the head region in an increasing percentage of patients as the pulse duration was increased

The present study completes the series of stimulation studies (Johansson 1969 a, b, and c) These studies deal with the effect of the stimulus parameters, peak current, pulse duration and pulse repetition frequency on the clinical threshold responses obtained by stimulation of a human subcortical area They have shown that the pulse duration influences the size of the projected localization area of the responses The nature of the responses depends on the pulse repetition frequency d At the stimulation threshold, the peak current value, on the other hand, has no observable definite effects on either the character or the projected localization of the responses within the intensity ranges tested

The reason for electrical brain stimulation in connection with stereotaxic surgery is to make sure that the electrode is not situated in unintended structures, the destruction of which might result in undesired loss of vital brain functions However the criteria of stimulation responses which could guarantee a successful therapeutic effect of surgery are not known with certainty It is probably of no great importance which stimulus parameter values are used when electrical stimulation is employed as a locating aid, provided they result in responses of some sort Since the clinical responses evoked from one and the same target area in this investigation were shown to be different for different parameter combinations however, it seems advisable always to use known standard parameter values for the test stimulations Otherwise it will be difficult to compare the responses from one patient to the next

If, on the other hand one intends to study the functional anatomy of the brain by means of electrical stimulation it seems impossible to conclude much about it unless pulses of many different repetition frequencies and durations are applied

The results of this and previous studies (Johansson 1969 a b and c) are discussed further elsewhere (Johansson 1968)

Conclusions

Electrical square wave pulses with a constant peak current and repetition frequencies ranging from 1 to 200 pulses/sec were applied to a human ventrolateral subventrolateral thalamic target area The clinical threshold responses evoked by the shortest effective pulses were recorded They consisted of motor and sensory" responses projected to the head and extremities of the contralateral half of the body

When the pulse repetition frequency was increased the pulse duration had to be correspondingly decreased in order to maintain a threshold stimulation. Plotted on double logarithmic coordinates, the curves showing the relationship between the two parameters had in the majority of the patients, a configuration approaching a straight line between 1 and 50 pulses/sec With pulse repetition frequencies of 100 and 200/sec, the threshold pulse duration was in most patients shorter than could have been expected from the inclination of the first part of the curves

The character of the threshold responses depended on the pulse repetition frequency used One and 5 pulses/sec evoked only muscular twitches, 10 and 20/sec resulted in tremor but also in sensations mostly described as electricity In the majority of the patients higher repetition frequencies resulted in 'sensory' responses only

The projected localization in the body of the responses changed as a result of the changing threshold pulse duration *i e* with increasing pulse duration an increasing number of patients presented responses projected to the head neck region

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Acta physiol. scand. 1969. 75. 476—483

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Effect of Acid in the Duodenal Bulb on Gastric Secretory Responses to Insulin Hypoglycemia[1]

By

GORAN NILSSON

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Abstract

NILSSON, G *Effect of acid in the duodenal bulb on gastric secretory responses to insulin hypoglycemia* Acta physiol scand 1969 *75* 476—483

Gastric acid secretion was stimulated by insulin hypoglycemia in Pavlov pouch dogs with isolated pouches of the duodenal bulb Perfusion of the duodenal bulb with 0 1 N HCl abolished the secretory response to a low dose of insulin (0 2 I U per kg b w), whereas the responses to higher doses (0 4 and 0 8 I U per kg b w) were only partially inhibited It is suggested that the bulbar mechanism inhibits secretory responses to insulin hypoglycemia by interference with the stimulatory action of gastrin

Previous studies indicate that the mechanism by which acid in the duodenum inhibits gastric acid secretion is located in the duodenal bulb (Andersson, Nilsson and Uvnas 1965 and 1967) Acidification of the duodenal bulb effectively inhibits the secretory response to a test meal (Andersson and Uvnas 1961) and to exogenous gastrin (Andersson *et al* 1965 and 1967) The present series of experiments was undertaken to determine the effect of acid perfusion of bulbar pouches on gastric secretory responses to vagal activation induced by insulin hypoglycemia

Methods

Surgical procedures

Five mongrel dogs, weighing 13—16 kg were provided with mucosal septal pouches of the stomach (Pavlov type) and innervated pouches of the duodenal bulb (Fig 1) according to a technique described by Andersson and Uvnas (1961) After each surgical procedure and before the experiments were started the dogs were allowed a period of 2—3 weeks for recovery

Experimental procedures

The experiments were started in the morning after the animals had been fasted for 18—24 hrs Basal acid output was recorded for 1 hr before gastric secretion was stimulated by a single intravenous injection of 0 2, 0 4 or 0 8 I U of insulin (Vitrum) per kg b w The acid output was collected every 15 min The volume was measured and the acidity was determined by

[1] A preliminary report of this investigation was presented at the XIIth Scandinavian Congress of Physiology, Turku, 1966 *Acta Physiol scand* 1966 *68* Suppl 277

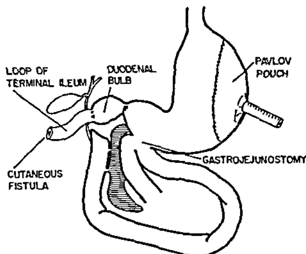


Fig 1 Surgical preparation of the dogs

titration with 0 01 N NaOH, using phenolphthalein as indicator The blood sugar level was determ [illegible] ghout the experiments by [illegible] perfused with 0 1 N HCl [illegible] the bulbar pouch against [illegible] as started simultaneously [illegible] 1 or 1 1/2 hrs The pH of the effluent perfusate collected during each 15 min period was determined It varied between pH 1 1—1 4 with acid and between pH 6 8—7 3 with saline A more detailed description of the technique of bulbar perfusion was given in a previous paper (Andersson *et al* 1967)

Results

The effect of bulbar acidification on secretory responses to insulin hypoglycemia was studied in 30 expts on 5 dogs Perfusion of the duodenal bulb with acid abolished the secretory response to 0 2 I U of insulin per kg (Table I Fig 2) Secretory responses to 0 4 and 0 8 I U of insulin per kg were partially inhibited (Table II and III, Fig 2) Perfusion of the duodenal bulb with saline did not significantly influence the secretory response to 0 2 I U of insulin per kg (Table I)

Determinations of blood sugar concentrations performed in control experiments and bulbar perfusion experiments show that the blood sugar level is not influenced by acid perfusion of the duodenal bulb

Discussion

Recent studies show that bulbar acidification inhibits the gastric acid response to a test meal (Andersson and Uvnas 1961) to low (Anderson, Nilsson and Uvnas 1965 1967) and to high (Andersson and Nilsson, to be published) doses of exogenous gastrin The present results demonstrate that acid perfusion of the duodenal bulb also inhibits secretory responses to vagal activation by insulin hypoglycemia

Intraduodenal infusion of acid has previously been reported to cause a certain

TABLE I Secretory responses to 0 2 I U of insulin per kg b w in Pavlov pouch dogs with and without perfusion of the duodenal bulb

Experimental procedure	Number of expts	Secretion in meq acid (mean and range): Control period (1 hr)	Response to 0 2 I U of insulin per kg b w: 1st hr	2nd hr: $^1/_2$ hr	2nd hr: $^1/_2$ hr	3rd hr
			Dog 1			
Controls	3	0 01 (0— 0 01)	1 85 (1 67— 2 14)	0 99 (0 61— 1 30)	0 12 (0 05— 0 22)	0 03 (0 02— 0 04)
0 9 % NaCl in the duodenal bulb during the 1st and 1st half of the 2nd hr	3	0 09 (0 02— 0 13)	1 54 (1 09 — 2 40)	0,73 (0 56— 0 97)	0 14 (0 07— 0 18)	0 05 (0 04— 0 06)
As a percentage of the mean for controls		—	83	74	117	140
0 1 N HCl in the duodenal bulb during the 1st and 1st half of the 2nd hr	3	0 (0— 0 01)	0 07 (0 02— 0 14)	0 03 (0 01— 0 06)	0 01 (0— 0 02)	0 02 (0 02— 0 03)
As a percentage of the mean for controls		—	4	3	8	67
			Dog 2			
Controls	2	0 02 (0 01— 0 02)	2 56 (2 50— 2 62)	0 77 (0 35— 1 18)	0 19 (0 07— 0 30)	0 03 (0 02— 0 04)
0 1 N HCl in the duodenal bulb during the 1st and 1st half of the 2nd hr	2	0 01 (0— 0 02)	0 10 (0 07— 0 13)	0 04 (0 02— 0 05)	0 01 (0— 0 01)	0 —
As a percentage of the mean for controls		—	4	5	5	0
			Dog 5			
Controls	4	0 01 (0— 0 02)	1 63 (0 44— 2 55)	1 15 (0 12— 2 15)		0 08 (0 06— 0 11)
0 9 % NaCl in the duodenal bulb during the 1st hr	4	0 01 (0— 0 05)	1 86 (1 04— 2 72)	0 74 (0 13— 1 60)		0 14 (0— 0 36)
As a percentage of the mean for controls		—	114	64		175

Table I continued

0 1 N HCl in the duodenal bulb during the 1st hr	4	0 02 (0— 0 04)	0 04 (0— 0 11)		0 01 (0— 0 01)	0 06 (0 01— 0 19)
As a percentage of the mean for controls		—	2		1	75
		Dog 19				
Controls	4	0 16 (0 08— 0 28)	1 72 (0 84— 3 75)	0 57 (0 13— 1 34)	0 35 (0 06— 1 01)	0 37 (0 02— 1 08)
0 1 N HCl in the duodenal bulb during the 1st and the 1st half of the 2nd hr	4	0 01 (0— 0 02)	0 02 (0— 0 07)	0 04 (0 01— 0 05)	0 05 (0— 0 17)	0 08 (0— 0 16)
As a percentage of the mean for controls		—	1	7	14	22
		Dog 20				
Controls	3	0 03 (0— 0 04)	0 83 (0 68— 0 97)	0 50 (0 42— 0 63)	0 13 (0 07— 0 18)	0 07 (0 04— 0 10)
0 1 N HCl in the duodenal bulb during the 1st and the 1st half of the 2nd hr	3	0 03 (0 02— 0 04)	0 03 (0 — 0 06)	0 01 (0 — 0 01)	0 (0 — 0 01)	0 02 (0 01— 0 04)
As a percentage of the mean for controls		—	4	2	0	29

Bold face figures indicate the presence of HCl in the bulbar pouch

degree of inhibition of insulin stimulated gastric secretion (Pincus *et al* 1944 Code and Watkinson 1955 Andersson 1960) Those studies were however not suited to elucidate the significance and the nature of the duodenal mechanism in inhibiting vagally induced gastric acid secretion The experiments of Code and Watkinson (1955) were performed on dogs in which the acid gastric content could enter the duodenum and probably activate the inhibitory mechanism also in the control experiments Pincus *et al* (1944) and Andersson (1960) perfused the duodenum through tubes inserted into the proximal portion of the duodenum Since the acid could flow freely down the duodenum adequate acidity was probably not attained in the duodenal bulb for optimal activation of the inhibitory mechanism Furthermore in the studies of Pincus *et al* (1944) and Watkinston (1955) gastric secretion was produced by the administration of very high doses of insulin (1—2 I U per kg b w) The importance of the dose used will be discussed below

Inhibition evoked by acid in contact with the bulbar mucosa may be mediated by humoral and nervous mechanisms of inhibition Recent studies indicate that the

TABLE II Secretory responses to 0 4 I U of insulin per kg b w in Pavlov pouch dogs with and without perfus on of the duodenal bulb

Experimental procedure	Number of expts	Secretion in meq acid (mean and range)				
		Control period (1 hr)	Response to 0 4 I U of insulin per kg b w			
			1st hr	2nd hr		3rd hr
				1/2 hr	1/2 hr	
		Dog 5				
Controls	5	0 07 (0 — 0 22)	2 67 (2 00— 3 05)	3 20 (2 60— 4 28)		0 23 (0 03— 0 64)
0 1 N HCl in the duodenal bulb during the 1st hr	5	0 10 (0 — 0 20)	**0 92 (0 31— 1 53)**	0 48 (0 15— 1 12)		0 13 (0 03— 0 35)
As a percentage of the mean for controls		—	**34**	15		57
		Dog 20				
Controls	2	0 08 (0 02— 0 13)	1 63 (1 28— 1 97)	2 43 (2 13— 2 73)	1 38 (1 37— 1 38)	1 54 (1 34— 1 73)
0 1 N HCl in the duodenal bulb during the 1st and the 1st half of the 2nd hr	3	0 04 (0 — 0 11)	**0 54 (0 41— 0 77)**	**0 26 (0 13— 0 38)**	0 05 (0 02— 0 11)	0 23 (0 12— 0 36)
As a percentage of the mean for controls		—	**33**	**11**	4	15

Bold face figures indicate the presence of HCl in the bulbar pouch

mechanism of inhibition is humoral Acid perfusion of the duodenum inhibits secretory responses to gastrin in gastric pouches which have undergone both parasympathetic and sympathetic denervation (Andersson 1963) Moreover acidification of totally denervated pouches of the duodenal bulb effectively inhibits secretory responses to exogenous gastrin (Nilsson to be published) Since bulbar acidification inhibits secretory responses to exogenous gastrin the inhibitory mechanism probably interferes with the stimulatory action of gastrin at the acid secreting glands As a matter of fact, it is not necessary to postulate a nervous mechanism of inhibition to explain secretory inhibition induced by acid in the duodenal bulb

It now seems well established that vagal impulses release gastrin from the antrum (Uvnas 1942, Pe Thein and Schofield 1959 Fyro 1967) Since antrum resection

TABLE III Secretory responses to 0 8 I U of insulin per kg b w in Pavlov pouch dogs with and without perfusion of the duodenal bulb

Experimental procedure	Number of expts	Secretion in meq acid (mean and range)				
		Control period (1 hr)	Response to 0 8 I U of insulin per kg b w			
			1st hr	2nd hr		3rd hr
				1/2 hr	1/2 hr	
Dog 5						
Controls	5	0 05 (0 — 0 15)	2 86 (2 32— 3 49)	4 12 (3 01— 5 43)		1 25 (0 26— 1 85)
0 1 N HCl in the duodenal bulb during the 1st hr	4	0 (0)	**0 87** (0 33— 2 15)	2 24 (0 41— 3 66)		0 94 (0 08— 2 00)
As a percentage of the mean for controls		—	30	54		75
Dog 20						
Controls	2	0 04 (0 — 0 07)	0 55 (0 21— 0 88)	2 65 (2 33— 2 96)	1 36 (1 33— 1 38)	2 25 (1 47— 3 03)
0 1 N HCl in the duodenal bulb during the 1st and the 1st half of the 2nd hr	2	0 04 (0 02 — 0 06)	**0 16** (0 14— 0 18)	**1.33** (0 72— 1 93)	0 91 (0 78— 1 04)	0 64 (0 56— 0 71)
As a percentage of the mean for controls		—	29	50	67	28

Bold face figures indicate the presence of HCl in the bulbar pouch

abolished the secretory response to vagal stimulation Uvnas (1942) suggested that gastrin is essential for the HCl glands to secrete optimally during vagal activation This observation was confirmed by subsequent studies Antrum resection markedly reduced the acid response to electrical vagal stimulation (Linde 1950) to sham feeding (Noring 1951 Olbe 1964) and to low doses of insulin (Olbe 1964) However there is also evidence indicating that under certain experimental conditions vagal excitation alone may activate the acid secreting glands Thus it has been shown that prolonged and intense stimulation of the vagi (Babkin and Schachter 1944) or high doses of insulin (Pevsner and Grossman 1955 Olbe 1964) stimulate gastric secretion even after resection of the antrum and the duodenal bulb Together these observations emphasize the necessity of considering quantitative aspects when vagal stimulants are used to evoke gastric acid secretion

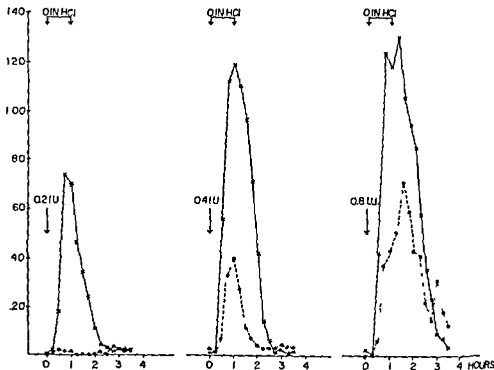


Fig. 2. Effect of bulbar acidification (pH 1.1—1.4) on secretory responses to insulin hypoglycemia produced with 0.2, 0.4 or 0.8 I.U. of insulin per kg b.w. Each curve represents the mean of 4—5 expts.

In the present study gastric secretion was activated by the injection of 0.2, 0.4 or 0.8 I.U. of insulin per kg. Secretory responses to the lowest dose of insulin were completely inhibited and responses to the two higher doses partially inhibited.

It was shown by Olbe (1964) that secretory responses to 0.2 I.U. of insulin per kg were abolished, whereas 0.4 and 0.6 I.U. of insulin per kg still produced considerable acid responses after surgical resection of the antrum and the duodenal bulb. There is a similarity between the effects of surgical resection of gastrin releasing areas as performed by Olbe (1964) and of bulbar acidification on secretory responses to low and high doses of insulin. Thus, both bulbar acidification and surgical resection abolished the secretory response to a low dose of insulin. From these observations, it seems reasonable to assume that bulbar inhibition interferes with and surgical resection eliminates gastrin necessary for the activation of the HCl glands. However, despite bulbar acidification or surgical resection higher doses of insulin still produced substantial responses. In my view, it might be possible that high doses of insulin induce such an intense direct excitation of the HCl glands that the glands become

activated without the co-operative action of gastrin. If so, the inability of the bulbar mechanism to completely inhibit acid responses to high doses of insulin in the present study indicates that the bulbar mechanism does not suppress the stimulatory influence of vagal excitation on the HCl glands but inhibits secretory responses to vagal activation by interference with the stimulatory action of gastrin.

This investigation was supported by research grants from Statens Medicinska Forskningsråd (W 30a), Svenska Sällskapet for Medicinsk Forskning and Torsten and Ragnar Soderbergs Stiftelse.

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Acta physiol. scand. 1969. 75. 484—495

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Effects of Hyperosmolarity on the Volume of Vascular Smooth Muscle Cells and the Relation between Cell Volume and Muscle Activity

By

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Abstract

ARVILL A, B JOHANSSON and O JONSSON *Effects of hyperosmolarity on the volume of vascular smooth muscle cells and the relation between cell volume and muscle activity* Acta physiol scand 1969 75 484—495

-ctrical and mechanical responses of vascular smooth muscle to variations in extracellular molarity were analysed in a previous study using isolated preparations of rat portal vein (Johansson and Jonsson 1968) The purpose of the present investigation has been to measure the changes in the volume of the cells of this smooth muscle when exposed to hyperosmolarity and to elucidate further the previously postulated relation between cell volume and muscle activity Distribution of water urea ^{14}C and sucrose ^{14}C in the isolated portal vein has been determined Urea ^{14}C rapidly equilibrates in a volume of 77 9 ml/100 g wet tissue weight which agrees satisfactorily with the total water content of the muscle (79 0 ml/100 g) The smooth muscle cells thus show a high membrane permeability for urea When the muscle is incubated in isoosmotic Krebs solution the distribution volume of sucrose ^{14}C reaches 45 4 ml/100 g within 15 min and then increases very slowly to 49 4 ml/100 g within 120 min The former value has been taken to represent the extracellular space After 20 min in a hyper osmotic medium (normal Krebs solution +150 mmoles sucrose/l) there is a decrease of total wet tissue weight to approximately 86 per cent of the control value and simultaneously a relative increase of extracellular space from 45 4 to 48 5 ml/100 g Calculations show that the intracellular fluid volume has then decreas ... osmotic behaviour of the smooth muscle ... The changes in muscle activity observed d ... sucrose Krebs solutions are discussed in th ... course of the uptake of urea ^{14}C and sucro ... relation between cell volume and activity in vascular smooth muscle

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Experimental alterations in the tonicity of the blood produced by infusion of hypo- or hypertonic solutions are known to cause marked changes in peripheral blood flow (for ref see Mellander *et al* 1967) The recent observation that exercise hyperemia in skeletal muscle is associated with an increase in the osmolarity of the regional venous blood led to the suggestion that increased tissue osmolarity could play a causal role in this physiological vascular response (Mellander *et al* 1967 Gray Lundvall and Mellander 1968) Vascular effects of variations in tissue osmolarity

have been shown to influence autoregulation of blood flow in skeletal muscle (Stainsby and Fregly 1968)

Studies on isolated vascular smooth muscle have been performed in this laboratory in order to elucidate the cellular mechanisms by which the tone of the vessels is altered by the osmotic changes These experiments which have been performed on preparations of rat portal vein have shown that variations in extracellular osmolarity affect the electrical and mechanical activity as well as the conductile properties of the vascular smooth muscle (Mellander *et al* 1967, Johansson and Jonsson 1968, Johansson and Ljung 1967)

The data obtained in our recent study (Johansson and Jonsson 1968) indicated that a relationship may exist between the volume of the smooth muscle cells and their level of activity in such a way that shrinking of the cells would be associated with hyperpolarization and inhibition swelling with depolarization and increased activity All effects on the muscle activity produced by various manipulations of *tonicity* could be fit into this scheme if it were assumed that the smooth muscle fibers behaved as osmotic cells with membranes permeable to water and urea but relatively impermeable to sucrose and ions

The purpose of the present experiments was to examine the validity of these assumptions *1* by *determining the distribution of urea* ^{14}C *and sucrose*-^{14}C *in the* portal vein after variable periods of incubation, *2* by analysing, in the light of these studies of tracer uptake, the changes in muscle activity that occur during and after exposure to hyperosmotic solutions, and *3* by estimating the volume changes of the vascular smooth muscle cells under conditions of increased extracellular tonicity The results help to explain the effects of hyperosmotic solutions on the portal vein described in our previous paper (see also Fig 2 below)

A preliminary report of the present study was given at the Scandin[illegible] ology Meeting in Goteborg March 1968 (Arvill, Johansson and Jons[illegible]

Methods

Female rats of the Sprague Dawley strain were used The [illegible] and 250 g The rats were fed a commercial laboratory an[illegible] *libitum*

The dissection of the portal vein preparation has been [illegible] article (Axelsson *et al* 1967) but it should be mentioned t[illegible] was always cut open longitudinally to reduce diffusion d[illegible] facilitate the blotting procedure In experiments where the [illegible] was measured the muscles were pre incubated for a period [illegible] solution of the following composition in mmoles/l NaCl 1[illegible] 1 19 $NaHCO_3$ 15 5 KH_2PO_4 1 19 Glucose 11 5 After the [illegible] were transferred to 10 ml Erlenmeyer flasks contai[illegible] Erlenmeyer flasks were kept in a gyratory shaking bat[illegible] 120 min The incubation medium was either norm[illegible] tion produced by adding sucrose 150 mmoles/l to the [illegible] with a gas mixture of [illegible] O_2 and 4 % CO_2 for [illegible] the *pre incubation medium was bubbled conti*[illegible] the experiments and the incubation media were gas[illegible]

The incubation media contained labelled urea [illegible] Chemical Centre Amersham England Urea-^{14}C [illegible]

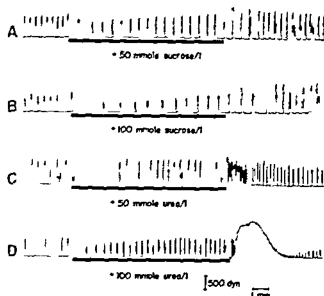


Fig 2 Effects of hyperosmolarity on spontaneous mechanical activity of rat portal vein Reproduced from an earlier report (Johansson and Jonsson 1968)

Fig 3 illustrates the mechanical responses seen in the present series of experiments on return to normal Krebs solution (arrows) after exposures, for different periods of time to a Krebs solution made hyperosmotic by the addition of 100 mmoles urea/l In record A the muscle had been exposed to the hyperosmotic urea Krebs solution for 1 min in B for 2 min in C for 4 min, in D for 6 min and in E for 12 min The excitation seen on return from hyperosmotic urea solution to normal Krebs was described in our previous article (Johansson and Jonsson 1968) and it was attributed to an osmotic swelling of the cells caused by the urea that had penetrated into the intracellular space during the exposure to the hyperosmotic

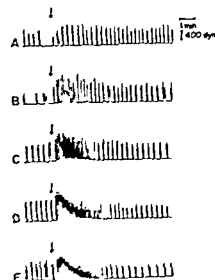


Fig 3 Mechanical responses of rat portal vein to normalization of osmolarity (arrows) after exposures for various periods of time to a Krebs solution made hyperosmotic by the addition of 100 mmoles urea/l. The muscle had been exposed to the hyperosmotic solution for 1 min in record A 2 min in B 4 min in C 6 min in D and 12 min in E respectively

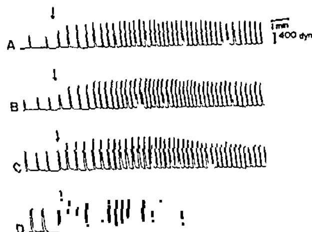


Fig 4 Mechanical responses of rat portal vein to normalization of osmolarity (arrows) after exposures for various periods of time to a Krebs solution made hyperosmotic by the addition of 100 mmoles sucrose/l The muscle had been exposed to the hyperosmotic solution for 6 min in record A 12 min in B 24 min in C and 48 min in D, respectively

solution It should be noticed that in Fig 3 there is a clear-cut increase in the degree of excitation from record A to D but apparently not from record D to E *i e* maximal excitation was obtained after an exposure to the hyperosmotic urea solution of approximately 6 min This is well in accordance with the abovementioned hypothesis because the distribution volume of urea ^{14}C has at that period of time almost reached its maximal level

Fig 4 illustrates a similar sequence of mechanical responses as Fig 3 but the muscles have here been exposed, for various periods of time, to a Krebs solution made hyperosmotic by the addition of 100 mmoles sucrose/l instead of urea The muscles had been exposed to the hyperosmotic solution for 6 min in record A 12 min in B, 24 min in C and 48 min in D It should be noticed that also in these experiments there is a phase of increased activity after return to normal solution but this excitation is delayed and less intense and it demands longer periods of preceding hyperosmolarity than in the case of urea The degree of excitation is clearly dependent on the duration of the exposure to hyperosmotic sucrose solution and does not seem to reach a maximum with the incubation periods used in Fig 4

3 Changes in the volume of the vascular smooth muscle cells

The following sets of experiments were performed in an attempt to estimate the volume changes of the smooth muscle cells produced by an increase in the extracellular osmolarity

The total tissue weight of portal vein preparations was determined at intervals using standardized procedures for blotting and weighing In the control period when the muscles were incubated in normal Krebs solution the values were satisfactorily reproducible The average of three values obtained for each preparation over the last 30 min in normal solution represents the control state in Fig 5 At time 'zero' the muscles were transferred to a Krebs solution which was hyperosmotic due to addition of sucrose 150 mmoles/l and the total tissue weight was determined after

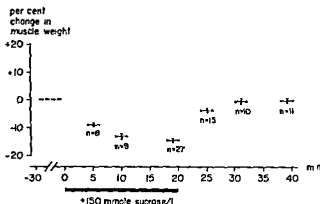


Fig. 5. Changes in the wet weight of isolated rat portal veins during and after exposure to a Krebs solution made hyperosmotic by the addition of 150 mmoles sucrose/l. The zero level represents the mean value of three determinations of the wet weight in normal Krebs solution. Vertical bars represent ± S.E.

variable periods of exposure to this medium as indicated in Fig. 5. The muscle weight decreased rapidly down to 85.8 ± 1.2 (S.E.) per cent of the control weight after 18 to 20 min in the hypertonic environment. No further significant decrease was found when longer exposure periods were used in a few experiments (not included in Fig. 5) and the routine was therefore to return the muscles to isotonic Krebs solution after 20 min. This led to a rapid recovery of tissue weight as illustrated Fig. 5.

When the Krebs solution was made hyperosmotic by the addition of urea instead of sucrose no significant reduction of total tissue weight could be measured at time intervals corresponding to those of the sucrose experiments in Fig. 5. Nor did we see any definite changes of tissue weight on return from hyperosmotic urea Krebs to normal solution.

Measurements of total tissue water were done on portal vein preparations incubated in normal solution and in hypertonic solution containing 150 mmoles sucrose/l. In a group of 13 muscles from normal medium the water content was 79.0 ± 0.26 (S.E.) per cent of the total tissue weight. The corresponding figures for 4 muscles incubated in the hypertonic solution for 20 min was 72.9 ± 0.52 per cent.

Finally the 'extracellular space' of muscles incubated in the hyperosmotic sucrose Krebs solution was estimated by studying the uptake of sucrose-^{14}C. The preparations were pre-incubated in normal medium for 30 min after dissection and then transferred to the hypertonic solution which also contained sucrose-^{14}C as in the control experiments presented under *I* above. The muscles were analysed after 2.5 and 15 min with regard to their sucrose-^{14}C content. The data are given in table II together with corresponding values for the uptake of sucrose-^{14}G of muscles in normal medium. It can be seen that the volume of distribution of the tracer per unit muscle weight is somewhat greater for the preparations incubated in the hypertonic solution. For the following calculations the distribution of sucrose-^{14}C in the muscles after 15 min is taken to represent extracellular space. As judged from the uptake curve presented under *I*, sucrose is completely equilibrated in the extracellular fluid after 15 min. On the other hand, it cannot be excluded that some

TABLE II Sucrose space of portal vein in normal Krebs solution and in hyperosmotic solution (normal Krebs + 150 mmoles sucrose/l)

Time of incubation in sucrose °C	Medium	n	Sucrose space ml/100 g Mean ± S E	p <
2 min	Normal	12	36.3 ± 0.91	0.01
2 min	Hyperosmotic	9	40.5 ± 1.11	
5 min	Normal	20	39.6 ± 0.71	0.001
5 min	Hyperosmotic	11	44.5 ± 0.75	
15 min	Normal	9	45.4 ± 1.29	0.025
15 min	Hyperosmotic	11	48.5 ± 0.49	

TABLE III

	Control Normal Krebs solution	After 20 min in normal solution + 150 mmoles sucrose/l
Total tissue weight g	100	85.8
Total tissue water g/100 g	79.0	72.9
Total tissue water g	79.0	62.5 (72.9 × 85.8/100)
Extracellular space ml/100 g	45.4	48.5
Extracellular space ml	45.4	41.6 (48.5 × 85.8/100)
Specific weight of extracellular fluid	1.007	1.025
Weight of extracellular fluid g	45.7 (45.4 × 1.007)	42.7 (41.6 × 1.025)
Weight of extracellular solutes g/100 ml (sum of solutes in respective media)	1.2	6.2
Weight of extracellular solutes g	0.5 (45.4 × 1.2/100)	2.6 (41.6 × 6.2/100)
Weight of extracellular water g	45.2 (45.4 − 0.5)	40.1 (42.7 − 2.6)
Weight of intracellular water g	33.8 (79.0 − 45.2)	22.4 (62.5 − 40.1)
Per cent decrease of intracellular water		33.8

entrance of sucrose into the cells has taken place over this period, as will be discussed below, and this would then lead to a slight overestimation of the extracellular space.

Table III summarizes the data presented in this section which are relevant for the calculations of the cellular volume changes elicited by the hyperosmotic solution containing 150 mmoles sucrose/l. For the sake of simplicity a mass of 100 g muscle in normal medium has been taken as a reference. The details of the calculations are given within parentheses in the table. The end result shows that 33.8 per cent of the intracellular water is lost after 20 min in the hypertonic medium when the total muscle weight seems to have reached a steady state (Fig. 5). Such water loss would increase the concentration of solutes in the intracellular fluid to approx

mately 51 per cent above the control value Since the addition of 150 mmoles sucrose to the normal medium implies an increase in extracellular osmolarity by about 50 per cent the calculated reduction in intracellular water indicates that the smooth muscle cells behave as almost perfect osmometers over the actual range of hyperosmolarities This refers to the fluid phase of the cells since the volume of the intracellular solids is unknown

The change of wet weight in the hypertonic solution which was measured directly in the experiments of Fig 5 can also be deduced indirectly on the basis of the determinations of total water content if due attention is paid to the change in the extracellular space and its solutes and if it is assumed that the absolute amount of other solids and solutes remains constant The latter calculation gives a reduction of wet weight in the hypertonic solution to 85 5 per cent of control which agrees satisfactorily with the measured value of 85 8 per cent

Discussion

The uptake of urea ^{14}C in the portal vein shows that this substance rapidly equilibrates in a volume which corresponds satisfactorily with the total tissue water thus indicating that urea easily penetrates the smooth muscle cell membranes A distribution volume of urea exceeding total tissue water has not been observed in the present experiments and the binding of urea reported for smooth muscle of frog stomach and dog intestine (Bozler 1961 Barr and Malvin 1965) thus does not seem to occur in the portal vein

The sucrose space after 15 min of incubation is restricted to about 45 ml/100 g muscle a volume which has been taken to represent the extracellular space of the preparation Values for extracellular space in different types of smooth muscle vary considerably with the tracer used for its determination (see *e g* Burnstock Holman and Prosser 1963 Bohr 1964 Barr and Malvin 1965 Goodford and Leach 1966) In principle the higher molecular weight of the test substance the smaller is its equilibration volume a finding which may indicate that the classical two-compartment model does not quite adequately describe the organization of the tissue (*e g* Bozler 1961 Barr and Malvin 1965 Page and Page 1968) It is possible that the slight increase in the distribution volume of sucrose between the 15 and 120 min periods of incubation in the present study may imply some entrance of the substance into an intracellular subcompartment Another possibility is that it represents diffusion into damaged cells

For the present estimations of the change in cell volume occurring in hypertonic solution it has been necessary to adopt the two-compartment model of the tissue and the measurements of extracellular space have been based on the uptake of sucrose ^{14}C The figure given above for extracellular space of portal vein in normal medium agrees with the higher values obtained in studies of other smooth muscle preparations (for ref see Burnstock Holman and Prosser 1963 Bohr 1964 The cell membranes seem to be essentially impermeable to sucrose and the results there

fore justify the previous assumption (Johansson and Jonsson 1968) that sucrose exerts a sustained osmotic influence on the smooth muscle cells whereas the effect of urea will only be transient

The time course of the decrease in wet weight on exposure to hypertonic sucrose Krebs solution is in accordance with the rapid diffusion of sucrose into the extra cellular space Calculations based on the changes in wet weight total tissue water and extracellular space showed that the intracellular fluid volume of the vascular smooth muscle in such hypertonic solution decreased quantitatively to an extent that should be expected from osmotic cells Shrinkage of smooth muscle cells in hyper tonic solutions has been demonstrated for frog stomach by Bozler (1959) and more recently for guinea pig taenia coli by Brading and Setekleiv (1968)

Our previous study (Johansson and Jonsson 1968) led to the suggestion of a relationship between cell volume and state of activity in the portal vein and the present investigation gives further support to this thesis as far as the influence of hyperosmotic solutions is concerned The short duration of inhibition seen on ad *ministration of* hyperosmotic urea Krebs *(Fig 2) is then explained by the fact that* urea cannot exert more than a transient osmotic influence due to its rapid entrance into the cells which is revealed by the uptake of urea ^{14}C (Fig 1) and by the ab sence of sustained decrease of wet weight in hyperosmotic urea Krebs Inhibition of spontaneous activity in the portal vein is maintained in solutions made hyperosmotic by the addition of sucrose (Fig 2) and this is well in accordance with the persistent shrinkage of the cells demonstrated by the present experiments A clear cut inhibi tion of activity remained even after very long periods of exposure (90 min) to such solution The fact that the inhibition is most pronounced immediately after the shift to the hypertonic medium when the cell volume is still changing *cf* Fig 2 and Fig 5) suggests that the efflux of fluid from the intracellular space adds in some way to the inhibitory influence of reduced cell volume

An immediate excitation is obtained on return to normal isoosmotic medium after a period of exposure to hyperosmotic urea Krebs solution (Fig 2 and 3) This re sponse was interpreted as a result of transient osmotic swelling of the cells caused by the amount of urea that had entered during the preceding period of hyperosmolari ty (Johansson and Jonsson 1968) Fig 3 above showed that this excitation was ab- sent when the muscle had been exposed to urea for only 1 min and as judged from the uptake curve of Fig 1 little urea enters the cells within such a short period The increase in excitation with gradually longer periods of preceding urea exposure (Fig 3) then runs parallel with the completion of the urea equilibration (Fig 1) These results are consistent with the previous interpretation that the increased ac tivity is associated with an osmotic swelling due to intracellular urea This swelling like the excitation would be of short duration due to the rapid diffusion of urea from the cells It should be noted however that in the present experiments no in crease in total tissue weight could be demonstrated in the period of excitation This may be due to the short duration of the swelling or it might indicate that other forces oppose the osmotic gradient to restrict the degree of swelling (Bozler 1962)

On return to normal solution after exposure to hyperosmotic sucrose Krebs there is also a phase of excitation before control activity is resumed (Fig 4) This excitation is, however, more sluggish in onset and less pronounced but of somewhat longer duration than the response seen after urea The excitation obtained after sucrose is also dependent on the duration of the preceding period of hyperosmolarity but, in contrast to the situation with urea, it grows slowly and does not reach an upper limit with the exposure periods used in this study If sucrose penetrates slowly into the cells the excitation might be attributed to a similar osmotic swelling as discussed for urea However the insignificant increase in the distribution volume of sucrose ^{14}C with incubation periods longer than 15 min (Fig 1) makes it doubtful that this mechanism alone could account for the clear cut progressive change in the degree of excitation seen for instance between C and D of Fig 4 An additional factor might be a gradual loss of intracellular ions due to the altered transmembrane concentration gradients produced by the sustained osmotic shrinkage of the cells The return to normal cell volume in isoosmotic medium would then be associated with for instance a subnormal ratio of $[K]_i/[K]$ which would lead to depolarization and increased phasic activity

In summary then the present results support the previous assumption that the spontaneous activity of the rat portal vein is correlated to the actual cell volume It has further been shown that the muscle cells behave like osmotic cells on exposure to hypertonic solutions The tissue responses in hypotonic solutions seem however to be somewhat more complex (Jonsson to be published) Besides the influence of the actual cell volume other factors seem to be of some importance for the changes in spontaneous muscle activity elicited by variations in osmolarity For instance the very *change* in cell volume as well as the secondary alterations in the concentrations of intracellular ions must be considered

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Evidence for a Hypothalamic Control of Renal Sodium Excretion

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Abstract

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Slow infusions (7.5 µl/min) of hypertonic NaCl into the 3rd brain ventricle of goats maintained on a NaCl-supplemented diet provoked natriuresis reaching maximum about 70 min after onset of the infusions. The natriuresis was less pronounced in animals receiving no dietary NaCl-supplementation. The magnitude of the natriuretic response was dependent on the molarity of the NaCl infused and on infusion duration. A much smaller relative increase in K excretion reaching peak values 20 min before maximum natriuresis also occurred. Aldosterone treatment did not prevent the natriuretic response and it could be elicited in diabetes insipidus animals showing that release of posterior pituitary hormones was not essential. Determinations of inulin clearance (C_{In}) indicated that glomerular filtration rate (GFR) increased and that relative reabsorption of Na decreased during the natriuresis.

Similar intraventricular infusions of hypertonic NH_4Cl resulted in greatly diminished renal Na excretion lasting for about 2 hrs. Such infusions also delayed by 90 min the normal natriuretic response to an intravenous NaCl load. C_{In} determinations indicated that a diminished GFR may have contributed to this reduction in renal Na excretion. The results indicate that renal Na excretion is under hypothalamic control in the goat. Possible mechanisms for this control are discussed.

It was previously shown (Andersson, Jobin and Olsson 1967 a) that injections of small amounts of hypertonic NaCl solution into the 3rd brain ventricle of the goat caused an increased renal sodium excretion, whereas injections of the equivalent amount of hypertonic NaCl into the lateral ventricle were ineffective in this respect. The natriuretic response to injections into the 3rd ventricle occurred also in the aldosterone treated animal. The present experiments provide additional evidence that an aldosterone independent control of renal sodium excretion is exerted from the vicinity of the 3rd brain ventricle.

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Methods

Animals Eleven female goats (b w 30 to 40 kg) were used for repeated experiments over a period of 9 months The animals were routinely confined in metabolism cages by means of collars and all experiments were conducted in these cages The goats had access to chaffed hay and water *ad lib* except in some of the infusion experiments when water was withheld In addition the goats were given 400 g of commercial grain mix each afternoon, preceded by 6 g of NaCl in some warm water To one of the goats the supplementary NaCl was not given during the first half of its experimental period

Brain implantations and infusion technique All animals were prepared with permanent cannulae in the anterior part of the third ventricle To avoid the disadvantage of having a "dead space" the infusions were performed via an inner cannula which was filled with the infusion solution and inserted to the ventricular end of the permanent cannula before the infusion was started The operation and infusion techniques were described in detail earlier (Andersson, Olsson and Warner 1967 b)

In all experiments the rate of intraventricular infusion was maintained at 7 5 μl/min The minimum interval between the experiments in each single goat was 3 days In all experiments made in 9 of the goats used for the present study (more than 90 % of the experiments) cerebrospinal fluid (CSF) was observed to drain out of the outer permanent cannula on compression of the neck both before insertion of and after removal of the inner cannula used for infusion of the salt solutions Thus a completely free mixing of the infused solution with the fluid of the 3rd ventricle was guaranteed in most experiments However in 2 animals the flow of CSF from the permanent cannula gradually became blocked Studies of the cannula placement after killing the goats revealed that in these instances the ventricular opening of the permanent cannula had made contact with the anterior wall of the 3rd ventricle Although this counteracted free drainage of CSF from the permanent cannula, it obviously did not prevent the infused solution from reaching the ventricle and mixing with the CSF

In addition to the ventricular cannula two goats were provided with a pair of thermocouple electrodes having their uninsulated tips placed bilaterally in the median eminence region At one stage during the experimental period these electrodes were used to produce a radiofrequency lesion in the median eminence region. The technique will be more fully described in a subsequent report (Andersson, Dallman and Olsson 1968)

Intravenous infusions Intravenous infusions of aldosterone or of inulin were made via a polyethylene tubing inserted into the jugular vein

Samples Blood samples were obtained in heparinized syringes from the jugular vein Urine samples were obtained at various intervals from a retention catheter inserted in the urinary bladder [illegible] d by slow in- [illegible] ained plasma [illegible] ntrations than [illegible] were chosen [illegible] attempt to minimize both washout and holdback effects At the end of each clearance period the bladder was washed two times with 10 ml water There was a quantitative return of the wash within 90 secs

Analyses Plasma and urinary Na^+ and K^+ concentrations were determined using an EEL flame photometer Urinary Cl^- concentrations were determined by the method of Brun (1949) Inulin concentrations in plasma and urine were determined by the Josephson and Godin modification (1943) of the diphenylamine reaction

Results

A Infusions of hypertonic NaCl into the 3rd ventricle

1) *Effects on urine flow and renal electrolyte excretion*

General characteristics Changes in electrolyte excretion which resulted from hypertonic NaCl infusions into the anterior part of the third ventricle were studied in 58 expts on 11 goats A general pattern of response to the infusions emerged In the first or second 10 min urine collection period after the start of an infusion urinary Na^+ concentration began to rise Peak concentrations (about 200 meq/l in goats maintained on extra dietary NaCl) were achieved between 50 and 80 min.

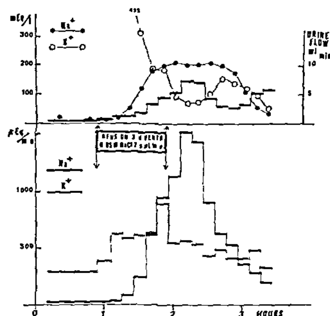


Fig 1 Renal effects of hypertonic NaCl infused into the 3rd brain ventricle

Hypertonic NaCl (0 85 M) was infused at a rate of 7 5 μl/min for 1 hr The goat (receiving dietary NaCl supplement) had free access to water and drank 3 6 l during the infusion period Note maximum Na^+ excretion at 70 min after the start of the infusion which is preceded by increased K^+ excretion The *onset of delayed water diuresis* resulting from the drinking to overhydration is seen at *3 hrs.*

arallel with the rise in Na^+ concentration, urine flow increased The result was a marked rise in renal Na^+ excretion rate which reached a maximum about 70 min after the start of an infusion Urinary K^+ concentration fell during the period of maximum natriuresis, but due to the increased urine flow an increased excretion of K^+ was always observed Maximum kaliuresis generally preceded peak Na^+ excretion by 10 to 20 min While there was usually a 10 fold increase in Na^+ excretion provoked by the infusions, K^+ excretion was seldom more than doubled or trebled Generally Cl excretion paralleled Na excretion, but occasionally the excretion rate was between those of Na^+ and K The renal response of a goat to an infusion of NaCl into the 3rd ventricle is illustrated in Fig 1 The large response reflects the facts that the salt supplemented goat was allowed free access to water during an infusion of 0 85 M NaCl which was continued for 60 min (see below) The mean

TABLE I Summary of the effects on renal Na^+ and K^+ excretion of hypertonic NaCl infused into the 3rd brain ventricle for 60 min

NaCl (0 85 M) was infused for 60 min (7 5 μl/min) in 18 expts on 7 goats Water was allowed *ad lib* in 11 expts and was withheld in 7 The results of all 18 expts were pooled and are presented as means ± S E M

	Na	K^+
Preinfusion excretion rate (μeq/min)	70 ± 10	220 ± 20
Maximal excretion rate (μeq/min)	730 ± 100	630 ± 40
Time of maximal excretion rate (mins after onset of infusion, calculated from 10 min periods of maximal excretion)	69 ± 2	52 ± 3

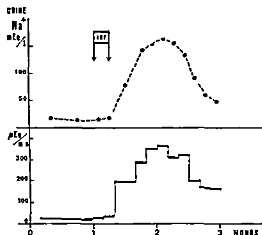


Fig 2 *Effects of a brief intraventricular infusion of hypertonic NaCl on urinary* Na^+ *concentration and renal* Na^+ *excretion*

Maximal Na^+ excretion occurred about 50 min *after* the infusion was stopped The salt supplemented goat was not allowed to drink during the experiment *INF* Infusion of 0 85 m NaCl for 15 min into the 3rd brain ventricle at a rate of 7.5 µl/min.

changes in Na^+ and K^+ excretion rates observed in all experiments in which this strong stimulus was applied are shown in Table I

Influence of varied experimental parameters Series of experiments were performed under different experimental conditions in order to elucidate whether the response of the goats was dependent on the osmolarity of the infused solution and on the duration of the infusion It was also studied in which manner drinking to overhydration and the sodium balance affected the response of the goats

In two goats (maintained on extra dietary salt) the responses to infusions of 0 35, 0.5 and 0 85 M NaCl of equal duration (45 min) were compared In these experiments the goats had free access to water and drank during the infusion period (Andersson *et al* 1967 b) Although there was no marked increase in water intake with increasing concentration of the infused solution, the magnitude of the natriuretic response increased with increasing molarity of the infused NaCl solution

Changes in the duration of the infusion period did not appreciably affect the interval between onset of the infusion and peak natriuresis Thus, after brief (15 min) infusion periods, maximal Na^+ excretion was not reached until about 50 min after cessation of the infusion (Fig 2) Series of experiments performed in 3 goats revealed that increases in the duration of the infusion from 15 min up to 1 hr also increased the magnitude of the natriuretic response However, preliminary experiments in another goat indicated that infusions for more than 1 hr (2 and 3 1/2 hrs) did not result in any further augmentation of maximal Na excretion rate Although the natriuresis was sustained, a gradual decrease was observed towards the end of the 3 1/2 hrs infusion period Free drinking during the infusion period invariably caused an augmentation of the natriuresis as illustrated in Fig 3

In one animal the effects of intraventricular infusions of hypertonic NaCl were studied before and after the goat had received supplemental salt (6 g NaCl/day) for a week. The animal had access to water during these experiments and drank rough-

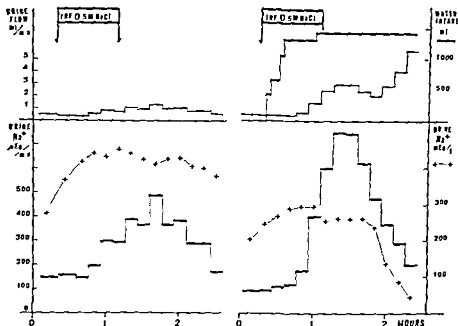
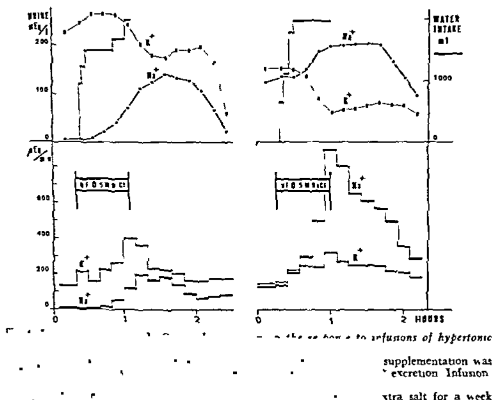


Fig 3 *Potentiating effect of drinking on the natriuresis caused by intraventricular infusion of c NaCl*

Water was withheld in the experiment shown to the *left*, whereas this goat had free access to water and drank during the infusion experiment shown to the *right* In both experiments 0 5 M NaCl was infused for 45 min at 7 5 μl/min

ly the same amount of water due to the infusions when maintained on the sodium-poor diet as it did after receiving the salt supplement The results of this study confirm earlier observations on the influence of sodium balance on the response to an elevated NaCl concentration in the 3rd ventricle (Andersson *et al* 1967 a) The basal urine flow of the goat maintained on the Na^+-poor diet was low and the Na^+ concentration was about 3 meq/l Consequently basal renal Na^+ excretion was very low (<3 μ eq/min) Intraventricular infusion of 0 5 M NaCl for 45 min caused an elevation of urinary Na^+ concentration to 155 meq/l 70 min after the onset of the infusion. With a simultaneous slight increase in urine flow, an almost 100 fold increase in renal Na^+ excretion was obtained, although the renal Na^+ excretion did not exceed 200 μeq/min A 3-fold increase in K excretion was seen Nevertheless in this experiment the absolute increase in K^+ excretion was greater than that of Na^+ excretion (Fig 4, left) The response of this goat to the corresponding infusion after it had received extra dietary NaCl for a week is shown to the right in Fig 4 Now the pre-infusion Na^+ excretion was above 100 μeq/min and the urinary Na^+ concentration over 100 meq/l The intraventricular infusion of 0 5 M NaCl caused a rise in urinary Na^+ concentration to 200 meq/l and a pronounced increase in urine flow resulting in a maximal natriuresis of 900 μeq/min i e a 5 times higher Na excretion rate than that observed in the corresponding experiment when the goat did not receive extra dietary salt.



... the response to infusions of hypertonic ... supplementation was ... excretion Infusion ... xtra salt for a week

In 2 expts aldosterone (Aldocorten, Ciba, 60 µg/hr) was infused i v for a period of 3 hrs During the intermediate hour hypertonic NaCl was infused into the 3rd ventricle The results confirmed earlier observations (Andersson *et al* 1967 a) that the administration of exogenous aldosterone does not prevent the natriuretic response to elevated NaCl concentrations in the 3rd ventricle

Effects of median eminence lesions After the response to intraventricular NaCl infusions had been tested, radiofrequency lesions were placed in the median eminence region of two goats A report on the technique and the influence of such lesions on the antidiuretic response to hypertonic solutions infused into the 3rd ventricle (Andersson *et al* 1967 b) will be given in a subsequent paper (Andersson, Dallman and Olsson) Relevant to the present report are two observations made in these animals In one goat the lesion was placed deep down in the median eminence and the development of diabetes insipidus indicated complete interruption of the functional connection between the hypothalamus and the neurohypophysis Subsequent intraventricular infusions of hypertonic NaCl performed both during the temporary and the permanent phases of diabetes insipidus, caused increased renal Na^+ excretion in spite of the apparent lack of ADH release

TABLE II Renal effects of hypertonic NaCl infusions into the 3rd brain ventricle The simultaneous determinations of inulin clearance (C_{In}), plasma Na^+ concentrations and renal excretion of Na^+ and K^+ were made in 4 expts on 2 goats Each animal was subjected

Urine collection period (mins)	—60 to —30	—30 to 0
C_{In} ml/min	69 ± 7	66 ± 6
Na^+ excretion rate μeq/min	79 ± 20	80 ± 20
K^+ excretion rate μeq/min	170 ± 30	160 ± 20
Filtered load Na^+ ($C_{In} \times P_{Na}$) μeq/min	9600 ± 1000	9400 ± 900
Filtered load K^+ μeq/min (C_{In} × estim P_K)	260 ± 30	250 ± 20
Na^+ fractional reabsorption as % filtered load	99 3 ± 0 2	99 2 ± 0 2
K^+ excretion as % filtered load	65 ± 10	65 ± 10

In the other goat the lesion was placed higher up in the anterior part of the median eminence and also involved the ependyma of the ventricular wall in the supraoptic region. The median eminence damage caused by this lesion was too small to induce diabetes insipidus In two experiments, performed within 10 days of the lesioning, both the natriuretic and thirst effects of intraventricular infusions of hypertonic NaCl had become dramatically potentiated In addition, the latency . for drinking and for the full development of the natriuretic response were u After the first 2 weeks post lesion, the response had returned to pre lesion intensity

2) *Effects on GFR and fractional tubular reabsorption of* Na^+

In 2 goats simultaneous determinations of C_{In} and renal Na^+ and K^+ excretion were made during 4 expts involving intraventricular infusions of hypertonic (0 85 M) NaCl The effects of one 30 and one 60 min infusion were studied in each animal Calculations of fractional tubular Na^+ reabsorption during each 30 min period of urine collection were based on C_{In}, plasma Na^+ concentration and Na^+ excretion In a similar manner, the relationship between filtered load and K^+ excreted was calculated

A summary of the results of these experiments is presented in Table II Using C_{In} as a measure of GFR, the infusions caused a mean increase in GFR of 20 % during the first 3 urine collection periods after the onset of the infusions (90 min) Na^+ excretion was highest 60 to 90 min after the start of the infusions (5 5 times higher than pre infusion levels) whereas maximal K excretion was observed in the preceding 30 min period (1 6 times higher than pre infusion levels) A decrease in fractional tubular reabsorption of Na occurred in conjunction with the increase in renal Na^+ excretion. The decrease was most evident during the period of maximal natriuresis (60 to 90 min) The absolute increase in K excretion was two times greater than the absolute increase in filtered K load indicating that the infusions had stimulated tubular secretion of this cation This effect appears to have been more accentuated in other experiments (cf Table I)

to one 30 min and one 60 min infusion (7.5 µl/min) of 0 85 M NaCl The results of these experiments are tabulated as means ± S E.M Infusions were started at time 0

0 to 30	30 to 60	60 to 90	90 to 120
81 ± 17	82 ± 3	81 ± 3	76 ± 5
280 ± 110	360 ± 60	380 ± 100	260 ± 90
260 ± 30	270 ± 30	240 ± 30	190 ± 30
11 600 ± 1500	11 400 ± 600	11 300 ± 600	10 600 ± 800
310 ± 40	310 ± 10	310 ± 10	290 ± 20
97 6 ± 0 9	96 8 ± 0.5	96 7 ± 0 9	97.5 ± 0 9
83 ± 15	87 ± 15	77 ± 9	65 ± 10

B Infusions of hypertonic NH_4Cl into the 3rd ventricle

1) *Effects on basal renal Na^+ excretion*

It was shown in earlier experiments (Andersson *et al* 1967 a) that, in contrast to injections of 0 85 M NaCl, injections of 0 85 M NH_4Cl into the 3rd ventricle of the goat neither caused drinking nor any increase in renal Na^+ excretion. However, brief intraventricular infusions of hypertonic NH_4Cl proved to be even more effective than infusions of hypertonic NaCl in eliciting an inhibition of the water diuresis in hydrated goats (Andersson *et al* 1967 b) On rechecking the records of these earlier experiments it became evident that the intraventricular injections and infusions of hypertonic NH_4Cl had in fact often been followed by a marked decrease in renal Na^+ excretion. Three of the present goats, in which free mixing of the infused solution with the cerebrospinal fluid of the 3rd ventricle was guaranteed (see methods), were used to explore this phenomenon further These animals were maintained on extra dietary NaCl and the effects of 15 to 40 min intraventricular infusions of 0 5 or 0 85 M NH_4Cl were studied In all experiments in which the control renal Na excretion was moderate or high (50 to 200 µeq/min) the infusions caused a reduced urine flow and a precipitous drop in urinary Na concentration. These effects were observed within 20 min of the onset of the infusion and resulted in a greatly diminished Na^+ excretion The full magnitude of the response often required one hr to develop The excretion rates for K and Cl were variable in the first 30 min but after 60 min these values generally had declined pre-infusion levels also Low electrolyte excretion persisted for about 2 hrs Then the excretion rate returned to control values No overshoot in Na^+ excretion occurred after the depression

In one goat C_{In} was determined in 3 expts in which the animal was subjected to 30 min infusions of 0 85 M NH_4Cl In one experiment the control pre infusion Na^+ excretion was very low (11 µeq/min) and the excretion was not significantly affected by the intraventricular NH_4Cl infusion In this experiment a rise in C_{In} over control levels was recorded after the intraventricular infusion In the two other experiments

pre-infusion Na^+ excretion was relatively high (about 150 µg/min). In these experiments the intraventricular infusion of NH_4Cl caused the usual depression of renal Na^+ excretion accompanied by an approximately 20 % decrease in C_{In}, indicating a reduced GFR (Fig. 5).

2) *Effect on the renal response to an intravenous load of NaCl*

Because intraventricular infusions of NH_4Cl caused a marked depression in Na^+ excretion, it was of interest to test the effects of such an infusion on the response to an i.v. load of hypertonic NaCl. The results of such an experiment might help to distinguish between central and peripheral control of Na^+ excretion in response to acute sodium loads. The effect of i.v. injections of 50 ml 0.85 M NaCl on urinary electrolyte excretion was studied in 3 similar experiments on one goat. In each experiment urinary Na^+ concentration increased steadily during the hour following the injection, as did urine flow. Maximal natriuresis was achieved 70 to 80 min after the i.v. injection (Fig. 6, top). The time sequence of the natriuresis following the i.v. saline load was thus very similar to that of the natriuresis produced by an intraventricular infusion of hypertonic saline in this animal (Fig. 1).

Alternating with these tests of the animal's normal response to i.v. injections of hypertonic NaCl, experiments were performed in which the same NaCl load was injected i.v. within 5 min after the start of an intraventricular infusion of NH_4Cl

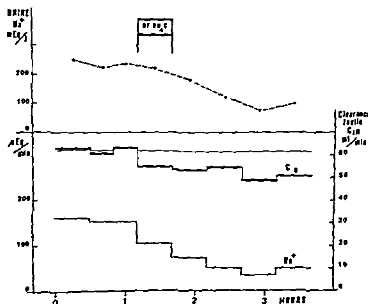


Fig. 5. *Renal responses to an infusion of hypertonic NH_4Cl into the 3rd brain ventricle.* NH_4Cl (0.85 M) was infused for 30 min at 7.5 µl/min. Note the progressive fall in urinary Na concentration and renal Na excretion. The simultaneous reduction in inulin clearance suggests that a reduced glomerular filtration rate may be part of the response.

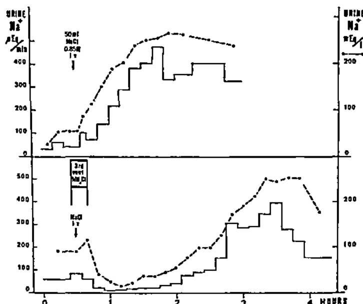


Fig 6 *Temporary reversal of the normal natriuretic response to a systemic saline load caused by a brief intraventricular NH_4Cl infusion*

Top The goat's normal natriuretic response to an i v injection of 50 ml of 5 % NaCl solution (NaCl i v *arrow*) Peak natriuresis is observed within 70 min of the injection

Bottom When the same i v injection of NaCl is made during a 15 min infusion of NH_4Cl (0 85 M, 7 5 µl/min) into the 3rd brain ventricle, the antinatriuretic effect of the intraventricular infusion dominates, and the renal response to the systemic saline load is delayed by 90 min

Dotted line=Urinary Na^+ concentration *Grey*=Renal Na^+ excretion

(0 85 M, 15 min) In spite of the large i v NaCl load, the infusions of NH_4Cl into the 3rd ventricle caused the usually conspicuous and long-lasting decrease in renal Na^+ excretion. This decrease was replaced by a normal natriuretic response which appeared 90 min later than in the control experiments (Fig 6, bottom)

3) *Other effects of intraventricular NH_4Cl infusions*

In addition to the renal effects described above, the infusion of NH_4Cl into the 3rd ventricle generally stimulated the appetite of the goats and induced polypnea together with peripheral vasodilatation (as indicated by a rise in ear skin temperature) In some experiments a mild sedative effect was also observed

Discussion

The quantity of Na^+ filtered into the proximal tubule per unit time is obviously an important factor in determining renal Na excretion rate. This has for example been clearly demonstrated in the dog by comparing Na^+ excretion from the two kidneys during unilateral reduction of GFR (Davidson, Levinsky and Berliner 1958) and is probably the case also in the goat (Beckett, Meyer and Dale 1967) It is generally

agreed that saline loading is accompanied by an elevation of GFR in most species including ruminants (Potter 1961, 1963) However, changes in tubular reabsorptive capacity can occur independently of variations in GFR and appear to play an important role in homeostasis The enhancement of Na^+ reabsorption by aldosterone in the distal part of the nephron is well established, and this hormone may also facilitate Na^+ reabsorption at more proximal sites (*cf* Thurau, Valtin and Schnermann 1968) Conversely, in response to some other factor tubular reabsorptive capacity for Na^+ may become reduced, causing increased natriuresis with no change in GFR This may occur in response to intravenous saline infusions and independently of changes in ADH and aldosterone secretion (Levinsky and Lalone 1963) Increases in GFR produced in the dog by means other than saline infusions have been found to cause little decrease in fractional Na^+ reabsorption whereas saline infusions leading to slightly smaller GFR increases, decreased fractional Na^+ reabsorption substantially (Lindheimer, Lalone and Levinsky 1967) Renal micropuncture experiments have provided further more direct evidence that a saline load causes a marked decrease in fractional tubular Na^+ reabsorption, and that this effect is independent of an elevated GFR (Dirks, Cirksena and Berliner 1965 and others) Transfusion experiments performed in the dog (de Wardener *et al* 1961, Johnston and Davis 1966) and in the cat (Cort and Lichardus 1963 a Lichardus and Pierce 1966) indicate this reduction in tubular reabsorptive capacity may be caused by a humoral acting as a natriuretic hormone or by dilution of an anti natriuretic hormone

From the present results it can not be said whether the natriuresis which occurs in response to infusions of hypertonic NaCl into the 3rd brain ventricle of the goat is simply due to an increase in GFR or whether it involves in addition a specific *effect on the Na reabsorptive capacity of the proximal tubule The natriuresis* induced by these infusions markedly resembles that observed in the goat and other species after systemic injections or infusions of NaCl showing a gradually increasing response with maximum development about 70 min after the onset of the stimulus (*cf* Fig 1, 2 and Fig 6 top) From determinations of C_{In} it appears evident that an increase in GFR occurs during the natriuresis but also that fractional tubular reabsorption of Na becomes reduced from >99 % to about 97 % of the filtered Na load (Table II) This decrease in fractional reabsorption of Na may simply reflect the limitations of the reabsorptive process to cope with the increased filtered load of Na^+ However the progressive increase in Na excretion in the presence of a rather constant elevation of GFR (Table II) suggests that a true reduction in tubular reabsorptive capacity may contribute to the renal response In view of the simultaneous increase in renal K excretion, the actual reduction of the fraction of filtered load reabsorbed by the proximal tubule is probably greater than Na^+ excretion rates would indicate In the animal receiving no extra dietary NaCl the infusion of hypertonic NaCl into the 3rd ventricle caused an absolute increase in K excretion which was even greater than that of Na excretion (Fig 4 left) Augmentation of renal kaliuresis invariably preceded that of Na excretion A similar in

crease in K^+ excretion has been observed in sheep during i v NaCl infusions and symptoms of K^+ depletion resulted from such infusions in animals maintained on rain water and receiving no extra dietary salt (Potter, 1968) The studies of Berliner and coworkers (*e g* Berliner, 1961) provide a rationale for the increase in K^+ excretion before and during the natriuretic response in goats and sheep Their work and the work of others make it evident that most of the filtered K^+ is reabsorbed in the proximal tubule Potassium secretion, occurring in a distal segment of the nephron is dependent on the availability of Na^+ for exchange Therefore the amount of Na^+ reaching the site of K^+ secretion may under certain conditions become the rate limiting factor determining K^+ excretion. The rapidly developing increase in kaliuresis observed during infusions of hypertonic NaCl into the 3rd ventricle may well be explained as due to stimulation of renal K^+ secretion which occurs when more Na^+ becomes available for exchange with K^+ at a distal site of the nephron The effect would be the same whether this Na^+ derives from an increased filtered load or from a reduced reabsorption of Na^+ in the proximal tubule, but this secretory K—Na^+ exchange is included in the calculations of fractional tubular reabsorption of Na^+ (Table II)

The daily sodium intake and the hydration state of the animals were factors which markedly influenced the magnitude of the natriuretic response to intraventricular NaCl infusions (Figs 3 and 4) Both dietary salt supplementation and drinking to overhydration in the beginning of an infusion period probably resulted in expansion of the extracellular fluid volume The daily sodium intake of ruminant animals maintained on chaffed hay and crushed oats is small and renal Na^+ excretion is very low (Andersson 1955, Dewhurst, Harrison and Keynes 1968) To judge from changes in plasma protein concentrations in the sheep (Potter 1968) and hematocrit in the goat (Andersson 1955) the extracellular fluid volume of these animals expands when they obtain an extra daily supply of NaCl Having free access to water the present goats usually drank 1 to 3 l of water during the first 15 min of the intraventricular infusion of hypertonic NaCl That this intake represented overhydration of the animals was indicated by a water diuresis which routinely occurred about 2 hrs after the onset of an infusion (the beginning of such a late water diuresis is shown at the far right in Fig 1) This long delay before the large water intake resulted in a water diuresis was apparently due to a stimulation of ADH release during the intraventricular infusion of hypertonic NaCl (Andersson *et al* 1967 b) and may well have resulted in an expansion of the extracellular fluid volume It has been shown in studies on sheep (Potter 1968) and dogs (Reinhardt and Behrenbeck 1967, Behrenbeck and Reinhardt 1967) that the magnitude of natriuresis and the increase in GFR produced by i v NaCl infusions are directly related to the initial magnitude of the extracellular fluid volume It seems likely that increased extracellular fluid volume also augments the natriuretic response to intraventricular infusions of hypertonic NaCl in the goat

Because infusions of hypertonic NaCl into the 3rd ventricle still produced natriuresis in a goat with diabetes insipidus and in animals treated with aldosterone

during the infusions, it can be concluded that neither increased secretion of ADH nor decreased aldosterone secretion is essential to the natriuretic response studied. This does not exclude the possibility that an infusion of hypertonic NaCl into the 3rd ventricle may depress endogenous aldosterone secretion in the goats It appears to be of great interest to study this possibility since the fundamental work performed in Australia in the sheep has provided evidence for central nervous system participation in the control of aldosterone secretion (*cf* Denton 1965) During the present study attempts were made to reveal the presence of a positive, salt-excreting factor in the natriuretic response by transfusing 2—3 % of the plasma volume from goats undergoing natriuresis to control animals To date the experiments have produced equivocal and inconclusive results (Andersson, Dallman and Olsson, unpublished results) Nevertheless, the time course of the development of a full natriuretic response was remarkably consistent and apparently independent of infusion duration, which suggests that there may be a humoral link in the natriuretic response However, the experiments do not help to distinguish between the secretion of a relatively slow acting "natriuretic hormone" and the inhibition of secretion and metabolic clearance of an "antinatriuretic substance"

The infusions of hypertonic NH_4Cl into the 3rd ventricle are of interest primarily because they produced renal effects which were opposite to those elicited by the intraventricular NaCl infusions (Fig 5) and because they delayed considerably the [illegible]al response to an i.v. load of NaCl (Fig 6) This provides evidence for a dien[illegible] control of renal Na excretion. The preliminary experiments involving determinations of C_{In} indicated that the NH_4 infusions resulted in a decrease in GFR but further experiments are needed to determine whether this is a consistent response and whether it is of essential importance for the observed marked decrease in renal Na excretion The fact that even brief (15 min) intraventricular infusions of NH_4Cl caused a depression of renal Na excretion lasting for about 2 hr suggests that a humoral "antinatriuretic substance" may be involved in the response A high concentration of such a substance present in the blood at the time of an i.v. NaCl injection would explain the observed long delay in the renal response to the i.v. NaCl load (Fig 6) From the present results no conclusions can be drawn about the mechanism of action of NH_4Cl infused into the 3rd brain ventricle Other effects than renal were observed making a nonspecific and unphysiological mode of action likely

The fact that an infusion of solute into the CSF of the 3rd ventricle elicits certain characteristic effects in no way permits definite conclusions to be drawn about the site of the sensitive brain tissue The infused solute can reach large areas of the brain by diffusion within the CSF throughout the ventricular system The transport of solute through the mesencephalic canal into the 4th ventricle is further facilitated by the continuous flow of CSF in that direction However, certain observations made during the previous and present experiments may justify the assumption that the neurons which react to the infusion of hypertonic NaCl to cause drinking release of ADH and natriuresis are located near the 3rd ventricle, probably in the hypo-

thalamus It was previously shown (Andersson *et al* 1967 a) that an amount of hypertonic NaCl which effectively elicited natriuresis when injected into the 3rd ventricle was ineffective when injected into a lateral ventricle of the same goat In the current study it was observed that the natriuretic response to infusions of hypertonic NaCl into the 3rd ventricle was markedly augmented during a 10 day period after the ependyma of the ventricular wall in the anterior hypothalamus had been damaged by radiofrequency heating Diffusion of Na^+ from the CSF into the hypothalamus proper may have been facilitated during this period of acute damage to the ventricular wall Support for hypothalamic involvement in sodium homeostasis has been presented by others Lockett (1966) found the presence of the posterior hypothalamus in donor cats essential to elicit normal natriuresis induced by saline injections into the perfusion system of isolated perfused cat kidneys, whereas Cort and Lichardus (1963 b) found that acute lesions in the posterior hypothalamus of the cat prevented the natriuretic response to bilateral carotid occlusion. The results of the present study suggest that variations in extracellular fluid volume rather than variations in extracellular Na^+ concentration *per se* may normally influence a hypothalamic mechanism of importance in the control of sodium homeostasis After diffusion into the surrounding part of the brain, the Na^+ infused into the 3rd ventricle would be expected to remain extracellularly and to cause a local expansion of the extracellular fluid If this expansion acts as the adequate stimulus to produce natriuresis it would provide an explanation for the fact that an i v injection of hypertonic NaCl, administered during an intraventricular NH_4Cl infusion, resulted in a delayed natriuresis developing at a time when plasma Na^+ concentration had returned to preinjection level (Fig 6, bottom) At this stage the depressive effect on Na^+ excretion of the centrally infused NH_4Cl had worn off, but the general expansion of the extracellular fluid volume caused by the intravenous NaCl injection remained as a possible stimulus to the hypothalamus to cause natriuresis

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Modulating Effect of Prostaglandin E_2 on Noradrenaline Release from the Isolated Cat Spleen

By

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Stimulation of the splenic sympathetic nerves in the dog and the cat has been shown to increase the outflow of prostaglandin from the spleen (Davies, Horton and Withrington 1967, Gilmore, Vane and Wyllie 1968) Recently prostaglandin E_1 was found occasionally to reduce the release of noradrenaline (NA) caused by stimulation of the splenic nerves in the cat (Hedqvist and Brundin 1969)

The present investigation was undertaken to study whether prostaglandin E_2 (PGE_2), one of the naturally occurring prostaglandins in the spleen is able to modify the release of NA from the sympathetic nerves of this organ

Cat spleens were isolated and perfused at a constant rate of 10 ml/min with a modified Krebs-Henseleit's solution as elsewhere described (Hedqvist and Stjarne 1969) About 1 hr before stimulation of the splenic nerves 40 μC of ^{3}H dl-NA was infused into the spleen The time course and profile of the NA output from the sympathetic nerves in the cat spleen was monitored by following the outflow of radioactivity in each 10 ml fraction of the effluent from the perfused organ while the quantitative determination of the NA outflow was based on fluorimetric assay of Na in pooled effluent fractions

Stimulation of the splenic nerves at a frequency of 10/sec for 20 sec resulted in a several fold increase of both radioactive and fluorimetrically determined NA in the venous effluent from the spleen In the presence of PGE_2 which was infused into the spleen at a molar concentration of 0 8—1 6$\times$10^{-6} for 5 min starting 3 min before the nerve stimulation, this response was consistently reduced often to less than 50 per cent of that of the preceding control stimulation (Fig 1) After cessation of the infusion of PGE_2 the NA overflow response to nerve stimulation gradually returned towards the preinfusion level Complete restoration of the response usually required 20 to 30 min Infusion of corresponding amounts of PGE_2 solvent (ethanol in saline) did not affect the NA overflow response to nerve stimulation

The present results clearly demonstrate that PGE_2 under the experimental conditions markedly inhibits the NA overflow response to nerve stimulation This effect appears to be the result of a decrease in the amount of NA actually released from the nerves rather than facilitated reuptake of NA released, since PGE_2 does

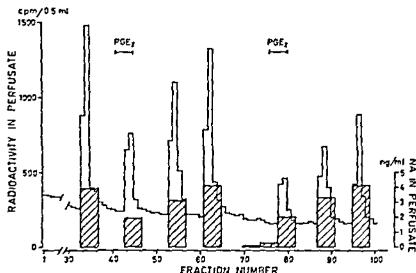


Fig 1 Perfused cat spleen loaded with ^{3}H-dl NA. Outflow of radioactivity and of fluorimetrically determined NA (shaded areas) from the spleen, resting and in response to nerve stimulation, 200 stimuli at 10/sec. Effect of PGE_2 molar concentration 1.6×10^{-6}

terially change removal of exogenous NA infused into the spleen (unpublished observation) The results suggest that PGE_2, locally mobilized by sympathetic nerve stimulation, may counteract further release of NA by a negative feed back mechanism, thus exerting a braking effect on the sympathetic neuro-effector system.

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Transient Transmembrane Water Movements in Crayfish Axons Detected by Transmitted Light Interference Microscopy

By

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Abstract

LIEBERMAN, E. M. *Transient transmembrane water movements in crayfish axons detected by transmitted light interference microscopy.* Acta physiol. scand. 1969. *75.* 513—517.

Transmitted light interference microscopy is shown to be a sensitive technique for detecting small transient changes in optical path differences of axons during and following electrical stimulation. It is tentatively concluded that water enters the axon during and following

... this may be a contributing factor to the optical properties of the axon during the excitation process.

Since 1949 several investigators (Hill and Keynes 1949, Bryant and Tobias 1952, Cohen and Keynes 1968 and Kayushin and Lyudkovskaya 1954) have observed changes in the optical properties of nerve bundles concomitant with nerve activity. In most cases these investigators have concluded that the optically detectable event represents alteration of axon volume as a result of excitation. Tobias (1952) suggested that Na^{+} and K^{+} exchanges during the excitation cycle cannot, alone, account for the volume change. A mechanism related to membrane structure, *i.e.*, water filled charged channels (Teorell 1955) may be important in this regard. Recent experiments suggest that electroosmosis (Stallworthy and Fenson 1966) and fixed charged properties of the axon surface (Lieberman *et al.* 1967) in fact may play an important part in steady state and transient electrical phenomena of excitable membranes.

Transmission interferometry was utilized for this study to detect changes in and measurement of optical path differences between an axon and its environment. From such measurements it is possible to determine preparation mass concentration and

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under certain conditions to interpret changes in mass concentration as changes in water content (Lieberman and Wright 1966) Transmission interferometry, in contrast to reflection interferometry as used by Kayushin and Lyudkovskaja (1954) and Sandlin *et al* (1968) which detects small movements of the surface of the preparation, may give more direct information concerning the role of water in nerve excitability

The results of experiments to be presented in this report represent preliminary studies of possible transient water movements through the membrane of isolated medial giant axons of the crayfish during excitation

Methods

The medial giant axon of the crayfish *Procambarus clarkii* was utilized for all experiments The dissection and isolation of the axon has been previously described in detail by Wallin (1967) A perspex chamber with a glass floor and coverglass top was designed for use with the Baker interference microscope The axon was continuously bathed in van Harreveld s (1936) crayfish physiological solution

The axon was longitudinally cannulated with a glass micropipette (10—40 Mohm tip resistance) for continuous recording of resting and action potentials In most experiments the axon was ligatured at the point of electrode entrance Potentials were detected with a high impedance preamplifier and monitored on an oscilloscope Massive platinum electrodes placed along the longitudinal axis of the axon were used for passing stimulating currents The frequency of stimulation and total number of action potentials produced were determined on an interval pulse counter

The Baker interference microscope (shearing system) either with interference contrast adjusted to even field or bright field conditions was used to view the axon In all experiments in which interference contrast was used the contrast was adjusted to midway between maximal and minimal brightness of the object the most sensitive position for detecting phase retardation changes A 150 W tungsten halogen lamp powered by stabilized DC power supply served as the light source Monochromatic light illumination (usually 644 nm) was obtained with an interference filter half bandwidth equal to 6 nm

Transient changes in light intensity as a result of shifts in phase retardation were detected with a silicon planar photo device (Texas Inst corp LS 400) placed in the image plane of an attached microscope camera The outputs of the photodevices were monitored by the oscilloscope or Ammeter depending on the nature of the experiment The photodevice output to the ammeter was also used to monitor and adjust field illumination to constant values by alteration of the substage diaphragm This was necessary to maintain a constant sensitivity and prevent saturation of the photodevice

The recording system had a maximum frequency response of one cycle per sec The sensitivity of the system was such that the smallest shift in light intensity that could be detected with certainty corresponded to a shift in phase retardation of 0 1° This represents a change of one part per 10^4 over the total phase retardation of the axon (approximately 415)

Results

Effect of high frequency stimulation

The following data represents the results obtained from five individual axons and could be repeated with each axon several times The optical responses were quite labile in amplitude and time course as was found earlier by Hill and Keynes (1949) As a result of these factors the author wishes to emphasize the preliminary nature of these experiments Traces in Fig 1 are from a single experiment and are exemplary of the optical responses obtained in several experiments

Fig 1 demonstrates the changes in phase retardation differences of the object seen after 1000 action potentials (traces in row I) at a stimulation frequency of 200

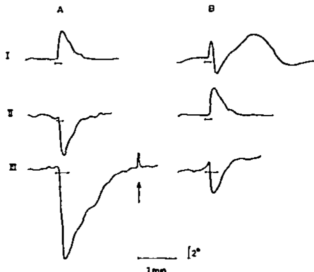


Fig 1 Optical interference and bright field responses from an isolated crayfish medial giant axon The horizontal bar under each trace represents the period of stimulation at 200 stimulations per second The time scale was the same for all traces See text for a complete description.

per sec The amplitude and time course of this response is increased with the number of action potentials at the same stimulus frequency (compare traces IIA and IIIA) On occasion it was possible to detect a bi or tri phasic response (trace I B) similar to the form of the opacity changes occasionally seen by Hill and Keynes (1949)

When the contrast of an object is adjusted midway between maximal and minimal an increase or decrease in phase retardation produces a positive or negative signal After readjustment of contrast setting also midway maximal and minimal lighting conditions but 180° advanced or retarded from the initial setting the sign of the signal will be opposite the previous one It is this characteristic which was utilized as the major prerequite for determining whether or not the signals recorded with interference contrast were due to shifts in phase retardation differences or from other optically detectable events *i e*, light scatter or light absorption The traces in row 2 of Fig 1 show that a shift of 180° of the contrast setting produces nearly identical but opposite sign signals It should be noted that the downward signal (II A) is of somewhat larger amplitude than its opposite (II B) and indicates the participation of other optical events Trace III A shows the response seen with interference contrast and trace III B a signal in bright field The low amplitude spike like deflection (at arrow in trace III A) was produced by momentarily decreasing illumination at the source and indicates that trace III B was produced by an increased light transmission through the axon likely as a result of transient decreased light scatter (Cohen Keynes and Hille 1968) It can be tentatively concluded that the signal as shown in III B as well as the signals detected in interference contrast were due to a decreased mass concentration (volume increase) A decrease in light absorption is an unlikely cause of the signal seen in trace III B since phase retardation changes could be detected with several wavelengths

It was found that these signals could only be obtained when action potentials were generated Subthreshold stimuli and abolition of action potentials with small amounts of KCl would also abolish the optically detectable events Axons which were damaged during preparation and thus would not produce action potentials also would not produce the optical signals, even in the presence of normal resting potentials (80—85 mV) When the light sensor was moved from the projected image of the axon or if the light source was interrupted optical responses could not be detected This rules out electrical coupling between the light detection system and axon potential recording system

Possible involvement of transmembrane hydrostatic pressure in optical properties of the axon

In some experiments in which the ligature around the axon at the point of electrode entrance was loosely tied (electrode could slip in and out of the entrance hole without stretching the axon) the previously described optically detectable events were not seen In one expt in which no ligature was placed around the axon no signal could be detected although in each of the cases action potentials were being continuously generated

Such experiments suggest that the optical responses are obtainable only when there is a hydrostatic pressure difference across the membrane

To test for the presence of transmembrane hydrostatic pressure measurements were performed on 4 axons A micropipette manometer for this purpose was developed for longitudinal placement within the axon The manometer resembled the glass microelectrode used in this investigation except that it was sealed at the large end The tip was drawn so that it had a 10—20 μ outside diameter and had no greater than a 70 μ outside diameter 5—8 mm from the tip The tip was filled with a potassium propionate solution for 2—3 mm from its opening trapping a large volume of air Changes in the length of the solution column were viewed with a dissecting microscope and measured with an ocular micrometer Each pipette was precalibrated for column length versus hydrostatic pressure and for the error produced by changing barometric pressure and temperature During each experiment barometric pressure and ambient temperature were continuously monitored Final values for hydrostatic pressure were corrected for these sources of error

In four measurements the hydrostatic pressure differential averaged 19 mm H_2O positive inside The individual values for each axon were 21.0 7.7 27.2 20.0 The zero pressure reference level was taken as the pressure (column length) which exists before the axon is ligatured at the manometer entrance hole These values compare favorably with these found by Baker Hodgkin and Shaw (1962) for squid axons

Discussion

The interferometric data on crayfish isolated axons are in accordance with the opacity data described by Hill and Keynes (1949 and are similar to the long term effects seen by Cohen *et al* (1968

That transient shifts in optical phase retardation can be detected indicates that water movements occur during and immediately following stimulation of the axon. These results are sufficient to explain the opacity changes (light scattering) seen by several investigaors on a variety of nerve and axon preparations In the most recent studies by Cohen *et al* (1968) light scattering changes were detected during the generation of a single action potential It is likely that the mechanism controlling this phenomenon is similar to that seen with tetanizing stimulation as presented here Initial experiments from this laboratory utilizing interference contrast and a detection system similar to that described by Cohen *et al* (1968) suggest that this is, in fact, the case

At the present time it can be only speculated as to the role that transmembrane hydrostatic pressure plays in the optical events described here but it can be said that a transmembrane hydrostatic pressure does exist and may be a contributing force for possible water transport during the excitation process Experiments which examine the effect of hydrostatic pressure, volume change by injection techniques and osmotic agents should greatly clarify the nature of water transport during excitation phenomena

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Acta physiol. scand. 1969. 75. 518—522

From the Institute of Zoophysiology, Uppsala Sweden

A Cyclic Variation in the Exfoliation of Cells from the Urethra of the Male Mouse

By

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Abstract

JÄRNEBRAND, K and J E KIHLSTROM *A cyclic variation in the exfoliation of cells from the urethra of the male mouse* Acta physiol scand 1969 75 518—522

Five different types of exfoliated cells representing different stages of cornification have been observed in daily urethral smears from male mice The frequencies of the cells belonging to of these types vary cyclically The mean duration of the cycles is about 3 5 days

The male sexual functions are subject to a cyclic variation corresponding to the oestrous cycle of the female animals This phenomenon was first observed in rabbits (Doggett 1956 Kihlstrom 1958) and has since been demonstrated to occur in cattle (Kihlstrom 1962) rats (Kihlstrom 1965) and possibly also in man (Doggett and Keilers 1962 Hornstein 1965, Månsson 1965) In all species studied so far the duration of this male cycle is approximately the same as that of the corresponding female sexual cycle A review of the literature within this field up to 1965 has been given by Kihlström (1966) In rats the male sexual cycle was studied (Kihlstrom 1965) by determining the frequency of spontaneous ejaculations (Orbach 1961) after preventing the animals from grooming their sex organs orally by means of a stiff girdle around the body Even isolated male mice ejaculate spontaneously (Orbach 1961) The small size and the high activeness of mice however make it very difficult to equip these animals with such girdles In rabbits it is possible to demonstrate the male sexual cycle by studying the frequencies of the cells of different types exfoliated from the mucous membrane of the male urethra (Kihlstrom and Hornstein 1964 a and b) This method seems suitable when studying whether or not there is a male sexual cycle also in mice

Material and methods

used for the experiments The animals
d care and temperature The experi
and daily urethral smears were prepared

at the same time of the morning during forty successive days One animal was excluded on account of a febrile infection, one on account of an insufficient exfoliation of cells and one for bleedings from the urethra

Smears were prepared by the following technique A thin polyethylene rod moistened at the

rapid drying The procedure seems to be quite painless for the animals After withdrawing the rod the moist smear was immediately spread on a clean slide and promptly fixed in a solution

microscopically in each preparation In 50 smears out of 920 the total number of cells was less than 200 in four of these even less than 100 (64, 73, 86, and 90, respectively)

The obtained frequencies of the different types of cells were calculated in per cent and the figures arranged in chronological series Consequently the interval between two successive figures is 24 hours Possibly occurring cyclic variations in these series have been studied by applying a serial χ^2 test related to the method of serial correlation as follows Within the chronological series mentioned above the data were divided into two classes The one class comprises the figures representing an increase compared with the preceding figures the other those representing a decrease The series was then displaced along its own copy, 1 2 3, etc time intervals After every displacement the number of data in the undisplaced series coinciding with data of the same class in the displaced series were noted The number of coinciding pairs thus obtained was then compared by means of the χ^2 test with the corresponding number calculated for a random distribution of the data of the two classes A cyclic variation if existing will manifest itself as a higher number of coinciding pairs than theoretically expected This will happen when the length of the displacement is close to that of the cycle or to a multiple of the latter

Results

Five different types of cells were found in the smears These cells are very similar to those described by Berthelsen (1958) and by Kihlstrom and Hornstein (1964 a) in urethral smears from male rabbits

The cells of type 1 and 2 are immature those belonging to type 3 intermediate between immature and cornified cells and those of type 4 completely cornified

Type 1 Medium sized cuboidal or somewhat elongated cells The cytoplasm appears darkly bluish green The ovoid nucleus is large and has a finely granulated chromatin The short diameter of the nucleus is longer than half the short axis of the whole cell

Type 2 Cells similar to those of type 1 but somewhat larger The short diameter of the nucleus ranges between a third and a half of the short axis of the whole cell

TABLE 1 Frequencies in per cent of different types of cells

Type of cells	Mean value ± SE	Extreme range
1	74 69 ± 0 56	8 5—99 0
2	3 55 ± 0 37	0 0—29 0
3	18 34 ± 0 47	1 0—69 5
4	2 22 ± 0 17	0 0 44 5
5	0 11 ± 0 01	0 0— 3 5

TABLE II. Statistically significant cycles regarding the frequencies of different types of cells.

Mouse no	Cells of type 1			Cells of type 3		
	Duration in days	χ^2	p<	Duration in days	χ^2	p<
1	Not significant			Not significant		
2	3	5.76	0.025	9	5.46	0.025
	6	4.25	0.05			
3	Not significant			3	4.56	0.05
				9	9.50	0.005
4	3	7.62	0.01	Not significant		
	6	16.66	0.0005			
	9	7.07	0.01			
5	3	8.76	0.005	9	5.12	0.025
6	3	7.27	0.01	3	11.92	0.001
				6	9.32	0.005
				9	7.07	0.001
7	Not significant			Not significant		
8	6	5.44	0.25	6	4.24	0.05
				9	6.46	0.025
9	3	11.92	0.001	9	3.90	0.05
	6	7.52	0.01			
	9	7.26	0.01			
10	3	9.36	0.005	3	7.82	0.01
	9	7.07	0.01	9	3.90	0.05
11	6	7.52	0.01	6	4.97	0.05
12	3	4.56	0.05	3	11.92	0.001
				6	7.52	0.01
				9	5.46	0.025
13	Not significant			9	5.46	0.025
14	3	11.25	0.001	Not significant		
	6	13.72	0.0005			
	9	11.40	0.005			
15	Not significant			Not significant		
16	6	6.80	0.001	3	6.08	0.025
				6	4.38	0.05
				9	9.54	0.005
17	Not significant			Not significant		
18	Not significant			Not significant		
20	3	4.56	0.05	3	11.70	0.001
	6	14.24	0.0005	6	19.58	0.0005
	9	9.32	0.005	9	9.32	0.005
21	2	23.68	0.0005	2	12.31	0.0005
	4	21.78	0.0005	4	10.68	0.005
	6	19.88	0.0005	6	10.85	0.001
	8	18.00	0.0005	8	14.05	0.0005
22	3	4.15	0.05			
23	3	9.76	0.005	3	7.62	0.01
	6	9.32	0.005	9	7.07	0.01
	9	14.22	0.0005			
26	Not significant			Not significant		

TABLE III Comparisons between the frequencies of cells of different types during the first 15 days and the last 15 days of the experimental period

Type of cells	First fifteen days	Last fifteen days	Comparison	
	Mean value ± SE	Mean value ± SE	t	P<
1	61.60 ± 0.94	86.25 ± 0.45	23.70	0.0005
2	5.44 ± 0.22	2.03 ± 0.09	14.21	0.0005
3	27.63 ± 0.82	11.05 ± 0.50	17.45	0.0005
4	4.97 ± 0.41	0.54 ± 0.05	10.37	0.0005
5	0.16 ± 0.02	0.08 ± 0.01	3.66	0.0005

Type 3 Large cells with a pale and vesiculated or dark and pycnotic nucleus The cytoplasm is stained lightly bluish green The diameter of the nucleus ranges from less than a third to about a tenth of that of the cell The cells are often folded with polyhedral outlines

Type 4 Cells of about the same size and shape as those of type 3 but with a yellow orange stained cytoplasm The nucleus is small and either dark and pycnotic or in an early stage of karyolysis Some cells have no nucleus others are pale and poorly stained as result of advanced cytolysis

Type 5 Cells as to size shape and appearance of the nucleus similar to those of type 1 or 2, but with a cornified yellow orange stained cytoplasm

The mean frequencies of these different types of cells are given in Table I As in rabbits the frequencies of the cells of type 1 and 3 were found to vary cyclically In 17 out of 23 animals there was a statistically significant cyclic variation as to the frequencies of at least one type of cells (Table II) In most cases the duration of the cycle seems to be about 3 days The observed statistically significant cycles with durations of 6 and 9 days might thus be multiples of the shortest cycle In one animal (number 21) the duration of the cycle seems to be 2 days with 4 6 and 8 days as statistically significant multiple intervals There are some reasons to suspect that in one animal (no 11) the mean duration of the cycles is 6 days and in one (no 13) 9 days The mean duration of the shortest cycles for all animals is 3.5 days with a range from 2 to 9 days As smears were taken only once a day the calculated length is impaired by a large error The mean duration of the oestrous cycle of the female mouse is 4.5 days with a range from 3 to 9 days (Asdell 1964 Albino mice however show a somewhat shorter cycle the mean length being 4.02 days (Asdell 1964)

During the experimental period there is a significant increase in the relative frequency of the cells of type 1 whereas the frequencies of the cells of the remaining types decrease significantly (Table III) This change might depend upon the irritation caused by the daily manipulation or indicate a continuous alteration with age

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Catechol-O-Methyltransferase in Autonomic and Sensory Ganglia of the Cat

By

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Abstract

Giacobini, E and S Kerpel-Fronius *Catechol-o-methyltransferase in autonomic and sensory ganglia of the cat* Acta physiol scand 1969 *75* 523—529

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The catecholamine inactivating enzyme catechol-o-methyltransferase (COMT) is present both in the central and peripheral nervous system (Axelrod *et al* 1959) It is however uncertain whether this enzyme is localized intra- or extraneuronally (Axelrod 1959, McCaman 1965, Alberici *et al* 1965) Recent microchemical studies on other enzymes (acetylcholinesterase cholineacetylase monamine oxidase) revealed two distinct groups of cells in the autonomic ganglia of the cat (Giacobini *et al* 1967, Buckley *et al* 1967 Consolo *et al* 1968) These cell populations differ in terms of catecholamine fluorescence (Hamberger *et al* 1965a) and the presence of related enzymes in individual cells

In the present paper the COMT activity of normal and preganglionically denervated sympathetic ganglia and of parasympathetic and sensory ganglia of the cat have been studied by the isotopic micromethod of McCaman (1965)

The aim of this study was to determine whether there existed a correlation of COMT activity with that of the monoamine oxidase (MAO) (Consolo *et al* 1968) and catecholamine content reported (Hamberger Norberg and Sjöqvist 196"

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the same ganglia Such correlation could suggest the presence of the enzyme in one of the two specific groups of cells previously characterized in the sympathetic ganglia of the cat, (Buckley *et al* 1967, Consolo *et al* 1968)

Material and methods

A *Preparation of the ganglionic material*

Adult cats of both sexes were anesthetized with Nembutal®, Abbott, 40 mg/kg i p Para vertebral, prevertebral and cervical sympathetic as well as parasympathetic (ciliary) and sensory ganglia (nodose cervical and lumbal spinal) were removed as quickly as possible and placed in physiological saline at 0° C After carefully removing the adherent connective tissue, under the dissection microscope with sharp forceps, the ganglia were weighed and homogenized in distilled water In order to disrupt the remaining collagenous capsule sur rounding the ganglia, a small amount of fine sand was used during homogenization The homogenate was centrifugated (MSE Mistral 6L centrifuge) at 2000 rpm at 0—2° C for 10 min The supernatant was either used immediately or stored at −70° C Since it was found that COMT activity rapidly decreases (to about 50 % of the original after 6 days) the ganglia were used in most cases within a period of 24 hrs Approximately 3 hrs were needed to isolate, clean, weigh and homogenize the complete series of ganglia

B *Denervation procedure*

The denervation of the lumbosacral ganglia (S_1 L_7 L_6) was performed under anesthesia according to the technique of Buckley *et al* (1967) The part of the ganglionic chain resected included L_4 and L_5 ganglia as well Denervated ganglia were assayed for enzyme activity 6—8 weeks after the operation

C *Enzymatic assay*

l the reagents were of analytical grade S adenosyl *L*-methionine (SAMe) (S methyl C^{14}) obtained from the New England Nuclear Corporation with a specific activity of 55 mC/mmole The determination of COMT activity was in principle similar to the method described by McCaman (1965) however some modifications were introduced

Buffer substrate solution was freshly prepared for each experiment with the following composition phosphate buffer pH 7 8 84 mM, 3 4-dihydroxybenzoic acid (protocatechuic acid) 1 1 mM $MgCl_2$ 5 4 mM SAMe 0 07 mM Ten µl of ice cold buffer substrate solution were added to 2 µl of homogenate in a pointed microtube having an internal diameter of 3 mm. The incubation was carried out at 38° C for 90 minutes and during incubation the tubes were sealed with rubber caps in order to minimize losses by evaporation The reaction was stopped by the addition of 2 µl of 2 5 N HCl followed by 60 µl of ethyl acetate After thoroughly mixing the samples were centrifuged (MSE Mistral 6 L centrifuge) for 5 min at 3000 rpm at 0—2° C in order to separate the phases 35 µl of the organic layer was transferred to a counting vial (20 ml and mixed with 0 75 ml of 0 3 M hyamine dissolved in methanol 10 ml of a solution containing 4 g PPO and 0 1 g dimethyl POPOP per liter of toluene were added The radioactivity was measured in a Packard liquid scintillation spectrometer and corrected for 100 % efficiency The results were expressed in umoles of product formed per g wet weight of tissue per hour

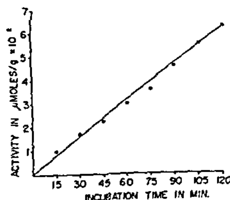


Fig 1 Time course of COMT activity by cat stellate ganglion homogenate (40 µg/µl) Each point represents the mean of 3 expts

TABLE I COMT, MAO activity and catecholamine content of autonomic and sensory ganglia of the cat

Type of ganglion	COMT activity		MAO activity[2]		Catecholamines[3]	
	Normal	Denervated	Normal	Denervated	A	NA
Superior cervical	0.064 ± 0.003[4] (11)	—	6.5 ± 0.3 (5)	—	0.12	9.7
Stellate	0.070 ± 0.003[4] (9)	—	6.6 ± 0.8 (6)	—	0.13	9.4
L_6	0.052 ± 0.005 (13)	0.051 ± 0.009 (6)	4.2 ± 0.4 (11)	4.1 ± 0.4 (7)		
L_7	0.059 ± 0.004 (14)	0.068 ± 0.005 (5)	4.2 ± 0.3 (13)	4.1 ± 1.1 (5)	0.80	6.8
S_1	0.047 ± 0.006 (12)	0.073 ± 0.009 (5)	4.0 ± 0.3 (13)	4.3 ± 0.4 (6)		
Coeliac	0.063 ± 0.015 (4)	—	11.1 ± 0.6 (6)	—	5.3	17.7
Superior mesenteric	0.052 ± 0.016 (4)	—	11.2 (3)	—	1.7	20.2
Inferior mesenteric	0.042 (1)	—	12.1 (2)	—	9.6	24.7
Ciliary	0.045 ± 0.005[4] (8)	—	2.05 ± 0.18[5] (12)	—	—	—
Nodose	0.068 ± 0.003 (11)	—	3.51 ± 0.28 (12)	—	—	—
Spinal	0.050 ± 0.005 (8)	—	1.47 ± 0.08[5] (16)	—	—	—

[1] COMT activity expressed in μmoles of product/hour/g wet

[2] MAO activity expressed in μmoles of product/hour g wet The values reported are from Consolo *et al* 1968 and Kerpel Fronius 1968

[3] K A Norberg and F Sjoqvist, Pharmacol Rev 1966 *18* 743—751 Means of 3 expts The data are expressed in μg/g A = adrenaline NA = noradrenaline

[4] The COMT activity of superior cervical and stellate is significantly (p < 0.02) higher than that of the lumbosacral ganglia and of ciliary lower than L7 (p < 0.02)

[5] The MAO activity of ciliary and spinal ganglia are significantly (p < 0.001) lower than that of *L7*

D *Control experiments*

Homogenates of cat brain and ganglia were a convenient source of enzyme for control experiments (Fig 1)

No activity was detected in the presence of homogenate which had been boiled for 30 min nor when the homogenate was omitted from the incubation mixture

E. *Statistical treatment of the results*

Since the percentage of "cholinergic" and "adrenergic" cells present in L7 could be on the basis of previous studies (Giacobini *et al* 1967 Buckley *et al* 1967 Cor 1968) this ganglion was selected as the reference for comparing the activity of

parasympathetic ganglia to sympathetic ganglia. The significance of the difference in the mean values of COMT and MAO activity (Table I) was tested according to the 't' of Student test

Comments on the method

The enzyme activity in cat brain and sympathetic ganglia was found to be rather low, therefore attempts were made to activate the enzyme or to increase the sensitivity of the method by lowering the blank values Different homogenizing media were tried these included 0 1 M phosphate buffer, pH 7 8 and isotonic glucose with NaCl (3 5 g glucose +0 3 g NaCl/100 ml water) The influence of various chemicals, chelating agents and detergents were also investigated e g mercaptoethanol, 5 mM, $MgCl_2$ 5 mM, EDTA 2 mM, Triton X 100 0 05 and 0 5 %, Cutscum 1 % The detergents produced a slight increase of enzyme activity but unfortunately these agents are not convenient for handling small volumes of homogenates since they produce a permanent froth Addition of BSA (bovine serum albumine, final concentration 10—20 $\mu g/\mu l$) in the incubation mixture exerted a protecting effect when the enzyme was present in low concentrations

The spontaneous decomposition of SAMe during incubation causes a marked increase in blank values Since SAMe is more stable at low pH the pH of the buffer substrate solution was lowered from 7.8 to 7 or 6 in some experiments pH 7 did not reduce the blank value significantly while pH 6 strongly diminished the enzyme activity as well

In order to decrease the blank values the volume of the buffer substrate solution was decreased to half that suggested by McCaman (1965), but the final concentration of the reagents in the incubation mixture was unchanged

Under the conditions described above the enzyme activity was found to be linear with the amount of homogenate added (20—80 μg) and with the time of incubation up to 120 min (Fig 1) Usually 40 μg of tissue was used In order to obtain a sufficient amount of the reaction product the system was incubated for 90 min In several control experiments, however the activity was the same after 60 or 90 min incubation

Results

The COMT activity of the sympathetic ganglia investigated is presented in Fig 2

Approximately the same level (0 042—0 070 μmoles/g/hour) of enzyme activity was measured in all the sympathetic ganglia investigated

Superior cervical and stellate ganglia however showed slightly higher values compared to those obtained for the lumbosacral ganglia ($p<0\ 02$)

The COMT activity in the ciliary ganglion was slightly lower than that of L_7 ($p<0\ 02$ Table I) No significant difference was found between the sympathetic and the sensory nodose and spinal ganglia (Table I) For comparison the MAO activity and the catecholamine content of several ganglia are reported in Table I It can be seen that the MAO activity of spinal and ciliary ganglia was significantly lower than that of L_7 ($p<0\ 001$)

Preganglionic denervation did not significantly change COMT activity of L_6 L_7 and S_1 ganglia The increase in activity observed for the L_7 and S_1 ganglion was not significant Due to the fact that S1 ganglia in the majority of cases are united the evaluation of denervation studies is difficult in these ganglia

Discussion

The first systematic study of the distribution of COMT activity in nervous tissue was carried out by Axelrod *et al* (1959 in the monkey In spite of considerable differences between the activities of different brain regions COMT activity could not be correlated to the noradrenaline level of the corresponding area No discrete

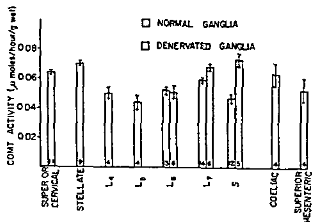


Fig 2 COMT activity in normal and preganglionically denervated sympathetic ganglia Vertical lines indicate ± S E. The numbers in the columns refer to the number of cats investigated

areas in the CNS have been demonstrated to contain only two types of neurons ("cholinergic and adrenergic), therefore any correlation of this kind may be rather difficult

From this point of view the sympathetic ganglia of the cat seem to be a more suitable tissue since up to now only two different neuronal populations have been identified (Giacobini *et al* 1967, Buckley *et al* 1967, Consolo *et al* 1968)

The availability of a sensitive micromethod (McCaman 1965) has made it possible to study COMT activity in single ganglia

Our results show that COMT activity in the sympathetic ganglia of the cat is almost equal and that the difference in COMT activity between sympathetic, parasympathetic and sensory ganglia is slight This suggests that COMT may be present in all three types of neurons (sympathetic parasympathetic and sensory) or in non nervous (glial) elements, or in both

Catecholamine level (Hamberger Norberg and Sjoqvist 1965a) has been correlated with MAO activity in various ganglia by Consolo *et al* (1968) and it was shown that these two parameters in the whole ganglia may be considered as a good indication of the number of ' adrenergic neurons as characterized by the presence of catecholamine fluorescence (Hamberger Norberg and Sjoqvist 1965a) and MAO activity measured in single cells (Consolo et al 1968)

The specific localization of MAO in adrenergic cells is also supported by the present results showing that MAO activity of ciliary and spinal ganglia is significantly lower (Table I) than that of L7 which contains predominantly adrenergic cells (Consolo *et al* 1968) A low level of MAO activity is to be expected in the ciliary and spinal ganglia as it is already known (Rexed and Euler 1951 Euler 1957) that sensory and parasympathetic nerves have very low content of catecholamines and that a moderate number of varicose nerve terminals coursing among the ganglion cells or surrounding the cell bodies in the sensory and parasympathetic (ciliary) ganglia of the cat fluoresce for catecholamines (Hamberger *et al.* 1965b Owman and Santini 1966)

The lack of correlation between COMT activity and either MAO activity or catecholamine level in the sympathetic ganglia suggest that the enzyme may not selectively be present in the "adrenergic" cells In this respect it is particularly striking that the prevertebral ganglia (coeliac, inferior and superior mesenteric) which show high catecholamine content and high MAO activity (Table I) have the same COMT activity as the other ganglia

Sympathetic denervation of the heart (Potter *et al* 1965) and immunosympathectomy (Iversen *et al* 1966) increases the proportion of O methylated derivatives from externally administered noradrenaline but does not decrease COMT activity This supports the idea that COMT is not confined to neurons

Only the direct measurement of COMT activity in isolated single nerve cells can give a definite answer to these questions However, although the present method (McCaman 1965) allows determination of COMT activity in 10—100 μg of tissue it is still not sensitive enough for measurement in single ganglion cells

Alberici *et al* (1965) reported that about 50 % of COMT is concentrated in the nerve endings fraction (synaptosomes) of the brain *i e* in neuronal processes In connection with these results we expected a decrease of activity after preganglionic denervation however this was not observed The fact that COMT activity remains unchanged after denervation does not completely eliminate the possibility that the enzyme is localized in the terminal processes since as in the case of MAO (Giacobini *et al* 1968) a variation in enzyme activity of the single cells may be masked by a corresponding change of activity of the presynaptic enzyme due to degeneration

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From the Department of Physiology Karolinska Institutet Stockholm Sweden

Unit Responses in the Cochlear Nucleus of the Rat to Pure Tones

By

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Abstract

MOLLER A R *Unit responses in the cochlear nucleus of the rat to pure tones* Acta physiol scand 1969 *75* 530—541

In a study of the response of single units in the cochlear nucleus of the white rat it was found that all the units which responded to pure tones showed a selectivity with regard to tone frequency Responses were obtained in the tone frequency range from 200 to 34 000 Hz Most the units responded to pure tones with a sustained train of discharges The threshold in se units was found to rise abruptly when the tone frequency was increased above that of the unit s characteristic frequency (CF) A much more gradual increase in threshold was noted when the tone frequency was decreased below the unit s CF The slope of the high frequency part of the tuning curves varied between 0 and 50 Hz per dB increase in threshold when measured 20—30 dB above the unit s threshold at its characteristic frequency The slope measured in that way showed no apparent dependence of on the CF of the units Many units with a high CF had tuning curves with a second peak located at a lower frequency A few units had almost symmetrical tuning curves with a slope of the same order of magnitude as that recorded for the low frequency part of the common type units The maximal obtainable firing rate of common type units was found to decrease rapidly when the frequency of the stimulus tone was increased above the unit s CF Conversely the maximal discharge rate was almost constant for tones below the unit s CF Iso-rate functions based on stimulus response curves were found to be similar to tuning curves when a discharge rate of about 20 per cent above the unit s spontaneous rate was used as a criterion of response

During the past 25 years considerable data have been collected concerning neural coding of auditory stimuli at the levels of the auditory nerve and the cochlear nucleus The great majority of these experiments has been performed in anesthetized cats using different types of microelectrodes (Galambos and Davis 1943 Galambos 1944, Greenwood and Maruyama 1965 Katsuki *et al* 1958 Katsuki Watanabe and Maruyama 1959 Kiang 1965 a b Kiang *et al* 1965 Moushegian Rupert and Galambos 1962, Pfeiffer 1966 Radionova and Popov 1966 Rose Galambos and Hughes 1959 Rupert, Moushegian and Galambos 1963 Starr 1965) Most studies dealt with such physiological characteristics as excitatory and inhibitory response areas to sinusoidal stimulation and temporal response patterns to pure tones and clicks In a few studies with like objectives other animals such as monkeys (Nomoto Suga and

Katsuki 1964), guinea pigs (Tasaki 1954, Tasaki and Davis 1955), bats (Frishkopf 1964) pigeons (Stopp and Whitfield 1961) and frogs (Frishkopf and Goldstein 1963) have been used The results of these latter studies were generally similar to those obtained in cats

The aim of the present study is to relate some psychoacoustic findings to electrophysiological data Thus this paper deals with 1) the electrophysiological thresholds to tone stimulation compared with that obtained in behavioural experiments and 2) the frequency selectivity of single units in the cochlear nucleus as a basis for the ability to discriminate small changes in tone frequency

Methods

In the present electrophysiological investigations more than 50 white rats of both sexes were used (additional rats were used for histological investigations) These animals ranged in weight from 240 to 260 g The rats were anesthetized with urethane administered intraperitoneally (400 mg), tracheotomized and canullated with a polyethene tube Both ear canals were cut close to the bone after which the pinnae were deflected The rat was placed in a headholder the hollo [illegible] The posterior part [illegible] was trepaned in t [illegible] arged by bone fore [illegible] observation of the cochlear nucleus The rat was placed in a box insulated from sound and vibration and its body temperature was maintained by usual measures Micropipettes drawn from Pyrex glass to tip diame [illegible] M KCl or 5 M NaCl served as [illegible] icroscopic inspection to obtain [illegible] de was inserted in the cochlear [illegible] silver electrode was placed in [illegible] outside of the sound insulated box by a hydraulic system The headholder was always maintained in the same position and the electrode was inserted in the same nearly dorso ventral direction thus assuring a certain uniformity of electrode placement The sound stimuli were delivered by a condenser microphone (Bruel & Kjaer type 4131) attached to the hollow earbars of the headholder (see Fig 1 A) A dc bias of 150 V was applied to the microphone (*cf* Møller 1964

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In a few experiments the sound pressure level near the eardrum was measured by a calibrated probe microphone (see Fig 1 A) Typical results of two such measurements are shown in Fig 1 B The sound pressure is given in dB re 0 0002 μb as a function of the frequency for 1 volt RMS into the earphone at 1 kHz This graph (1 B) shows the frequency response of the entire stimulus generating system including amplifiers and gate

The potentials led off from the microelectrode were amplified by a Grass P 6 amplifier The amplifier was ac-coupled as was the coupling between the electrode and the cathode follower Unit potentials were displayed on a monitor oscilloscope and recorded on one track of a two track tape recorder (Revox G 36 The other track was used for recording the synchronizing pulses the stimuli and voice comments

During the experiment the recorded discharges were made audible by means of a loud speaker When a unit was located its response area to sinusoidal stimulation was determined The stimuli were initially presented in 50 msec bursts at a rate of 6 sec while the sound intensity was set (usually in steps of 5 dB) The frequency of the tone was varied in order to determine the frequency at which the unit responded or failed to respond The criterion of response was a just noticeable increase in firing rate This procedure was done in the sound intensity range from the threshold of the unit to 50 dB to 80 dB above

When processing the recorded data the nerve impulses were high pass filtered in order improve the signal noise ratio (the cut-off frequency was usually in the region of 1000 and lead to a level discriminator which produced short rectangular pulses every time

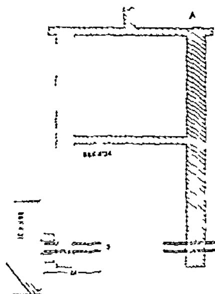
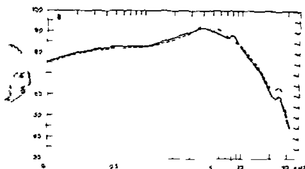


Fig 1 A Headholder with sound source (Bruel & Kjaer 4131 condenser microphone) and probemicrophone (Bruel & Kjaer 4134) The measurements are in millimeters



B Frequency response of acoustic stimulator Sound pressure in dB re 0 0002 μb measured near the ear drum in two experiments is shown for constant electrical input to the sound generating system. At 1 kHz the input to the condenser earphone was 1 volt RMS

level exceeded a preset value This trigger level was adjusted so that all the nervous discharges activated the trigger but not the background noise The setting of the trigger level was guided by a monitor oscilloscope on which the recorded signal was displayed The oscilloscope beam was intensified when the trigger was activated thus every event which activated the trigger device was marked with a bright spot on the oscilloscope To process those spikes two variable gates were installed in the counting circuitry which made it possible to select pulses which occurred during given time intervals after the initiation of each stimulus A modified Dawe digital counter tallied each spike as it occurred during the selected time interval

Results

The results reported in this paper are based on recordings from 170 units selected as being suitable for this present work out of a total number of 214 units All the units were obtained from the dorsal and ventral cochlear nucleus The remaining 44 units only responded to transient sounds and their response pattern will be described in another paper (Moller 1969 All the units included in this present study responded to tone bursts with an intensity slightly above threshold with a sustained train of discharges which lasted as long as did the tone In many cases satisfactory

recordings could be made for more than one hour and a few units were kept for more than four hours

The amplitude of the recorded potentials varied from less than 100 μV to 2 mV in the more than 200 units observed Potentials were initially positive and were either monophasic or diphasic In exceptional cases a response from more than one unit was recorded at the same time This superimposition resulted in spikes of different amplitude or shape

All units which responded to pure tones were selectively sensitive to tones of different frequency, *i e* there was always one particular tone frequency at which a particular unit exhibited its lowest threshold This has been termed the characteristic frequency, or CF of the unit by Galambos and Davis (1943) Some units were active in the absence of any intentional sound stimulus Such activity is here termed 'spontaneous activity' although this may possibly be a response to sound originating within the animal itself such as Browning Movements in the cochlea (see Koerber *et al* 1966) The rate of spontaneous activity was usually constant for a long time although in some cases the discharge rate changed during the recording without any known reason

When contact with a unit was established its response area to pure tones was determined by mapping its threshold as a function of tone frequency The resulting curve, the 'tuning curve (Kiang 1965 a), describes the units response area As expected the response area widens when the sound intensity was increased above the unit's threshold The shape of the tuning curves varied among different units but the great majority of units showed an abrupt rise in threshold for frequencies above the units characteristic frequency while a much more gradual increase was seen for frequencies below the unit's best frequency (CF)

The difference in slope between the low frequency and high frequency skirts of the tuning curves in the common type of units is often less pronounced for units which have their characteristic frequency in the range above 10 kHz In such units the threshold rises more abruptly below the unit s CF than it does for units which have their best frequency below 10 kHz This is illustrated in Fig 2 which shows a representative sample of tuning curves of the common type with characteristic frequencies distributed over the frequency range where response has been obtained

In the rat several tuning curves differed from the general shape and some had two instead of one peak (similar to what has been shown in the cat) The sensitivity of the low frequency peak was always lower than that of the high frequency peak although the low frequency peak was broader than the high frequency peak (when plotted on a logarithmic frequency scale) In accordance with previous findings in the cat (Rose *et al* 1959) rat units occasionally demonstrated a tuning curve which was much broader and more symmetrical than the common type as illustrated in Fig 2

In the following only units with tuning curves of the common type will be discussed

The threshold of 82 units determined at their characteristic frequency

Fig 2 Examples of tuning curves of 11 typical units belonging to the common type The acoustic stimuli were tone bursts (50 msec long) presented at a rate of 6 sec

Fig 3 The sound intensity in dB re 0 0002 μB required to produce an average increase of 20 per cent of the firing rate of spontaneously active units is shown as a function of the units characteristic frequency For units without spontaneous activity the threshold was defined as the intensity required to produce an average firing of 0 1 discharges per presentation of 50 msec tone bursts

The triangles connected by the dashed line in figure 3 are the mean values of the thresholds determined in psychoacoustic experiments on the white rat by Clack and Harris (1963) The lowest values of threshold given by Clack and Harris is about 20 dB lower

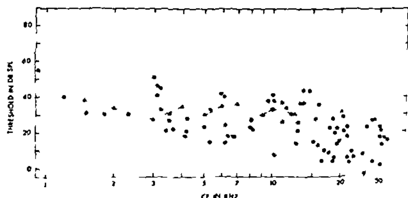


Fig 3 Threshold in dB re 0 0002 μb at the characteristic frequency of 82 units plotted as a function of CF Behavioural threshold is marked with triangles connected with dashed lines

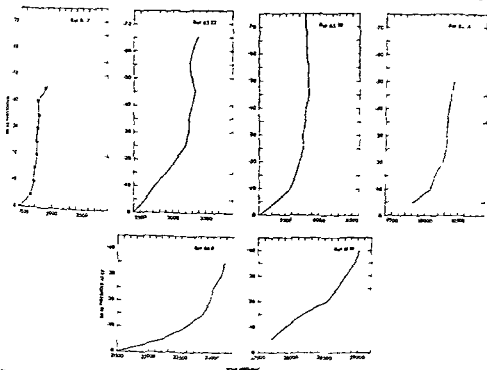


Fig 4 The high frequency part of typical tuning curves from units of the common type shown on a linear frequency scale to improve frequency resolution The threshold is given in dB relative to threshold at the characteristic frequency of the individual units The frequency scale in Hz of the graphs begins at the particular unit's characteristic frequency

At higher frequencies the electrophysiological thresholds are somewhat lower than those recorded by psychoacoustic means Between 20 and 30 kHz the threshold determined in the present study is remarkably low and in several cases was near the reference level (0 0002 μB)

In order to detect a small change in tone frequency on a spectral basis the auditory system presumably makes use of the change in relationship of excitation between units with different characteristic frequencies The largest change in excitation occurs when the frequency of a tone is located at the steepest part of a unit's tuning curve It is thus of particular interest to study the steep high frequency part of the tuning curves in more detail

The high frequency portion of some typical tuning curves of the common type is shown in Fig 4 Here these curves are plotted on a linear frequency scale The vertical scale gives sound pressure in dB above the unit's threshold at its characteristic frequency It is seen that the curves are steeper well above the threshold than they are near threshold At sound intensities 20 30 dB above the threshold at the unit's CF, the rise in threshold is often as much as 1 dB for an increase of 10 Hz In some units the slope may even become negative about 10 50 dB above threshold at the

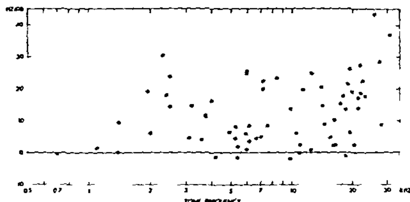


Fig 5 Slope values of the tuning curves (high frequency part) found for the common type units as a function of the unit s CF The slope was measured from 68 tuning curves in an intensity range from 20 dB to 30 dB above the unit s threshold at their characteristic frequency

CF This reversal implies that the frequency has to be decreased when the intensity is increased in order to retain a response Fig 5 depicts the slope in Hz per dB of the tuning curves measured in the intensity range between 20 and 30 dB above the unit s threshold at its CF The abruptness of the high frequency part of the tuning curves from a representative number of the common type units is clearly noted There are considerable individual variations in the magnitude of the slope and the diagram indicates that the slope of a unit s tuning curve is not related to the unit s CF in any specific way

The previously described characteristics of the unit responses refer to the thresholds of the units The next step in this study on the frequency discrimination of single units was to investigate the frequency selective properties *above* threshold The frequency dependent properties in the intensity range above threshold can be illustrated by curves showing the sound intensity required to produce a certain response as a function of sound frequency (iso-rate functions) Such plots are based on a number of *stimulus response curves obtained from stimulation with pure tones with frequencies covering the unit s response area* The response discharge rate was measured in response to stimulation with 50 msec long tonebursts (*cf* Rose *et al* 1959 Greenwood and Maruyama 1965)

It should be added that this method does not allow any differentiation between the onset response and the steady state response The latter may have a different rate of growth when the sound intensity is increased

A family of such curves (Fig 6) for two typical units relates the mean number of discharges per toneburst recorded during the first 55 msec after the onset of 50 msec tonebursts as a function of sound intensity Each intersect was based on 32 presentations

Most of the stimulus response curves (Fig 6 A were found to be monotone and S shaped reaching an asymtotic value at high sound levels Certain units showed

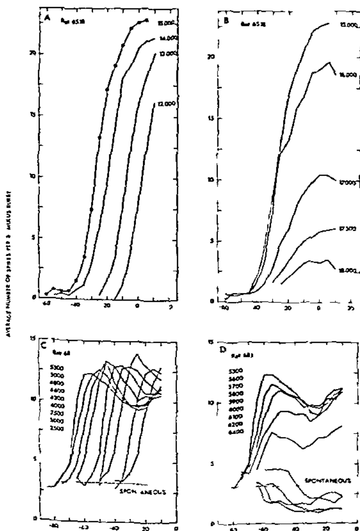


Fig 6 The response of two units to stimulation with pure tones of different frequencies is shown as a fun[illegible] character[illegible] The d ffe[illegible] figures T[illegible] of discha[illegible] duration is shown (The points of measurements are indicated on the upper left curve by filled circles.) The unit represented by the upper two graphs had no spontaneous activity while in the lower two graphs the approximate rate of spontaneous activity of that unit is indicated by the horizontal dashed line D also shows the average number of discharges evoked during 50 msec after the termination of the sound

stimulus response curves (Fig 6 C and D which had a peak at a certain sound intensty and a firing rate which decreased when the intensity was either increased or decreased from this value Units which were spontaneously active always showed a decrease in their firing rate immediately after the termination of the stimulus at

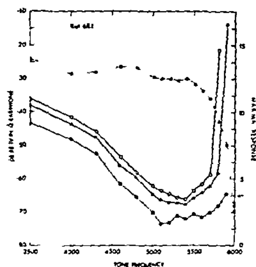


Fig 7

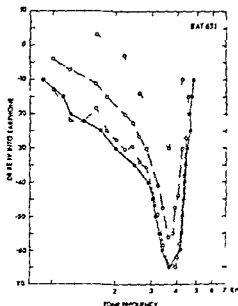


Fig 8

FIG 7 Iso-rate functions shown as a function of tone frequency The curves were constructed from stimulus response curves of the type illustrated in Fig 6 The individual curves show the *sound intensity required to produce an average number of discharge per tone burst of 4 7* (filled circles) 7 8 (triangles) and 9 4 (open circles) The spontaneous discharge rate of this unit was approximately 3 7 discharges (measured in the same way as the stimulated activity) (dashed line and right side scale) shows the maximal obtained spike count per stimulus burst

FIG 8 Iso-rate functions (open circles) of a unit compared with the tuning curve of the same unit (filled circles) These curves were obtained in the same way as those in Fig 7 and the individual curves represent the average number of discharges of 3 1 4 7 and 6 2 per tone burst. The unit s spontaneous discharge rate was approximately 1 5 discharges measured in the same way as in the stimulated activity

sound intensities somewhat above threshold It was generally found that the maximum obtainable firing rate decreased as the tone frequency increased above the characteristic frequency of the unit (Fig 6 B and D) Conversely the maximum discharge rate was almost constant in the frequency range below the unit s CF (see Fig 6 C) The middle part of the curves was also less steep for frequencies above the unit s CF than it was for frequencies below the CF (Fig 6 B and D

By means of stimulus response curves obtained at a number of tone frequencies iso-rate functions were computed Fig 7 illustrates typical curves for a unit with its CF of about 5400 *Hz* plotted on a linear frequency scale Such a plot gives a more detailed picture of the frequency-dependent properties of the units than do the previously shown tuning curves Fig 2 It is thus found that the response area of most of the units studied do not have a sharp peak but are rather flat in a narrow frequency region around the unit s CF The maximal firing rate is also shown in Fig 7 (dotted line) As was mentioned earlier the sharp decrease in the maximal obtainable firing rate above the unit s CF is evident from figure 7

Since a substantial amount of data is needed for construction of iso-rate functions only a limited number of units can be investigated that way The data from which the tuning curves are derived are easier to make and a large number of units can be studied Iso-rate curves from a typical unit are graphed in Fig 8 with the unit s tuning curve obtained in the same way as previously described It is seen that the tuning curve (filled circles) agrees well with the iso rate curve representing an average number of discharges per tone burst of 3 1 discharges (open circles) Only those discharges were counted which appeared during the stimulus duration During an equivalent length of time in the absence of stimulation the average spontaneous activity was about 1 5 discharges in this particular unit It is thus evident that the tuning curves do not express the threshold of the unit but rather the sound level required for a certain increase in firing rate

Discussion

The present study demonstrates that the shape of the response areas of units in the rat cochlear nucleus in response to tones is similar to that found in the cochlear nucleus of the cat (Galambos and Davis 1943 Greenwood and Maruyama 1965 Kiang 1965 Moushegian *et al* 1962 Rose Galambos and Hughes 1959) Several of these investigators (Galambos and Davis 1943 Moushegian *et al* 1962 Rose *et al* 1959) found that some units had response areas with a single peak while others had two peaks which is in agreement with what has been found in the rat However there are quantitative differences in that the highest sound frequency which elicited a response was 34 kHz in the rat compared to 48 kHz in the cat s eighth nerve (Rupert *et al* 1963)

There is probably a tonotopical organization of units in the rat cochlear nucleus similar to what has ben reported in the cat (Rose *et al* 1959) Although this has not been studied in detail low frequency units were often found near the lateral surface of the cochlear nucleus in the rat For practical reasons this region was not penetrated with the microelectrode as often as were the medial parts of the nucleus This sparse exploration may be one of the reasons why low frequency units are not found as often as are high frequency units Furthermore the distribution of neurons recorded from by a certain type of microelectrode may not necessarily be representative of the actual distribution of neuronal elements in a given part of the cochlear nucleus Such a bias may arise as one type of microelectrode may be more capable f recording from a certain type of neurone (Kiang 1965 b) Despite these disclaimers with regard to CF it is remarkable that so many units have their best frequency between 15 kHz and 30 kHz and that the threshold of these units is so low

Many previous investigators have stated that most units show an abrupt rise in threshold when stimulated with pure tones with a frequency above the unit s best frequency while a much less steep rise in threshold was seen when the tone frequency was decreased in the range below the unit s best frequency No estimatio the steepness of the flanks of the tuning curves has be

kopf (1964) and Rose *et al* (1959) Previous investigators published their results in the form of tuning curves plotted on a logaritmic frequency scale which does not permit the reader to estimate the value of the slope of the high frequency part of the tuning curves

Frishkopf (1964) using bats reported a slope of about 300 dB/octave of the high frequency part of tuning curves obtained in primary units of the auditory nerve The best frequency of these units was not reported by Frishkopf but he indicated that the range was 10 kHz to 70 kHz (at 10 kHz 300 dB per octave corresponds to 20 Hz/dB at 50 kHz it is 100 Hz/dB) The slope found by Frishkopf on bats is somewhat higher than those found in rats in the present study In addition the present study indicates that the slope is almost constant in absolute value over most of the rats auditory range It should also be emphasized that Frishkopf's results were obtained in recordings from primary fibers There may very well be a reasonable difference betwen the steepness of the tuning curves of primary units and that of cochlear nucleus units although Kiang (1965 b) reported that the general shape of tuning curves of the cochlear units are similar to those of the eight nerve

Units in the cochlear nucleus of the cat were shown by Rose *et al* (1959) to have a response area which only extended 0 2 octave above the unit s CF and was almost independent of the stimulus intensity The present findings on the rat agree well with those results on cats since it was found that the tuning curves in the rat ar nucleus have a rather flat region around the unit s CF and an abrupt rise in threshold above that region

The high frequency flanks of the tuning curves are much steeper than the mechanical filter function of the basilar membrane It has frequently been inferred that this sharpening of the mechanical resonance curves is the result of inhibition The quantitative data given indicate that the tuning curves are sufficiently steep to serve as a basis for frequency discrimination on a spectral basis without taking into account the coding of the periodicity of the sound in the train of discharges

The low threshold values of units with a CF between 10 kHz and the upper limit of responses (30 kHz) obtained with tones at the unit s CF (Fig 3) is somewhat surprising since the middle ear s transmission properties probably falls at a rate of at least 12 dB per octave in that frequency range (Møller 1965) The threshold values of the unit responses do not show such a decrease as a function of frequency On the contrary the responses show an almost constant sensitivity in that region In a recent study on the unit responses in the cat superior olive (Tsuchitani and Boudreau 1967) comparatively low threshold values has been demonstrated

It has been reported (Greenwood and Maruyama 1965 Rose *et al* 1959) that the stimulus response curves for different units in the cat s cochlear nucleus vary in shape and that the tuning curves obtained from one particular unit are different in shape for different tone frequencies The fact that the maximum obtainable spike rate decreases rapidly above the unit s CF (while it is almost constant below) is an interesting and unexplained phenomenon which must be regarded as a part of the unit s frequency selective properties

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Unit Responses in the Rat Cochlear Nucleus to Repetitive, Transient Sounds

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Abstract

MOLLER A R *Unit responses in the rat cochlear nucleus to repetitive transient sounds* Acta physiol scand 1969 75 542—551

The response patterns are described of cochlear nucleus units (termed rat transient units) which only responded to a tone burst with a single discharge evoked immediately after the onset When these cells were stimulated with bursts of repetitive clicks such units showed a marked selectivity to the click repetition rate At a low click repetition rate one discharge was evoked for each click but this one to one relationship failed rapidly when the click rate was increased slightly above a given click repetition rate Any additional increase in click rate resulted in only a single discharge fired immediately after the onset of a click burst and thus resembling these units response to tone bursts By replacing the click sounds by short bursts of pure tones presented repetitively it was shown that rat transient units were not selective to repetition rate *per se* but rather to the duration of silent interval between successive sounds The activity of these units to click stimulation could be inhibited by broad band noise or by pure tones within certain frequency limits Furthermore these units showed a spectral selectivity which appeared when the stimulation was pure tones or band pass filtered clicks The spectral region of highest sensitivity varied among the individual units in the range from 5 to 20 kHz and was thus widely different from the critical repetition rate for click stimulation The latter rate was found to vary from 200 to 800 clicks per second in different units

These findings may thus explain why in psychoacoustic experiments repetitive presentation of short sounds results in a pitch perception which is dependent on the length of the silent period *between* successive sounds

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It was recently found that certain units in the cochlear nucleus of the cat only respond to the onset of tone bursts (Kiang 1965 Pfeiffer 1966) Those authors named the neurons on units Similar units have been encountered in an electrophysiological study of the cochlear nucleus of the rat (Moller 1969) The aim of the present paper is to critically analyze the responses of these units to different types of repetitive transient sounds and to present new interpretations relating these neurophysiological data to certain psychoacoustical observations on pitch discrimination

Methods

The type of experimental animal (rat) and the technical procedures have been described previously (Moller 1969) In the present study the temporal pattern of unit activity is illustrated by a "dot display" (Haapanen *et al.* 1955) and by post stimulus time histograms…

In the dot display each spike is represented by a dot placed along a horizontal time axis which is triggered when the stimulus starts. The responses to succeeding stimuli are displayed in horizontal and parallel rows and then recorded on slowly moving film (see Fig. 1). Post stimulus time histograms were computed from the responses to 256 stimuli with the aid of a TMC "Computer of Average Transients" (type 400 A). The resolution of the time scale was equal to the channel width of the analyser and usually was set at 156 μsec. Each of the computer's 400 channels thus occupied 156 μsec. (The channel width is often named "bin width".) The results of this type of analysis were displayed on an oscilloscope and photographed. The readability of these displays was improved by superimposing a 5 kHz triangle wave on the vertical deflection of the oscilloscope. The resultant display so obtained consisted of lines instead of dots (cf. Fig. 2).

As to the stimulus, repetitive click sounds were generated by applying short rectangular pulses (duration 30 μS) from a Grass S4 stimulator to the condenser microphone (B & K 4131). The latter was used as sound source. A Wavetek type 116 generated a selected number of cycles of pure tones.

Results

On the basis of their response patterns, the recorded units (total number 214) in the rat cochlear nucleus could be divided into two groups. One group of units (170 cells—called *sustained units*) responded to continuous sounds with a sustained train of discharges which lasted as long as did the sound (when the sound intensities ranged from values slightly above to well above threshold). Another group of units (44 cells) only responded with a single discharge to stimulation with tone bursts. The discharge followed immediately after the onset of the stimulus. In the following these latter units will be called *transient units*. Both types of units showed a tone frequency selectivity. The transient units had a much higher threshold and broader tuning curves than the common sustained units. The transient units were found both in the dorsal and ventral cochlear nucleus with an apparant preponderance in the dorsal nucleus. None of the transient units showed any spontaneous activity.

The response pattern of transient units to stimulation with repetitive clicks (produced by applying 50 msec long bursts of rectangular pulses with a duration of 30 μS to the condenser earphone) revealed certain interesting properties. Fig. 1 shows the time pattern of the discharges to click stimulation of a sustained unit (left column) and that of a transient unit (right column). The click repetition rate is shown by inserted legend numbers. Each discharge is represented by a dot and each horizontal row of dots represents one presentation of repetitive clicks. As seen the discharges of the sustained unit fuse when the repetition rate is increased whereas each response of the transient unit correspond in time to the click stimuli. The failure to respond to clicks with high repetition rates of this type of unit is also evident. It is seen that the precise one to one relation between stimulus and response is first lost towards the end of the stimulus presentation when the click repetition rate is slightly increased above the range where this one to one relationship excist. When the click rate is further increased the unit fails to respond during successively larger segments of the stimulus duration until only a single spike remains at the beginning of each presentation.

The functional differences and similarities between the two types of units is further illustrated in Fig. 2. These "post stimulus time histograms" relate the tem

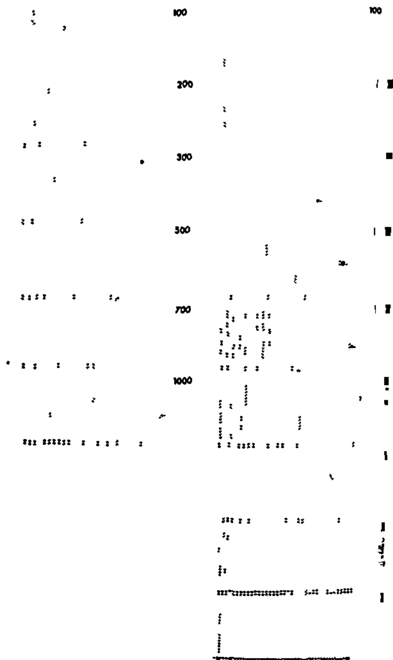


Fig 1 Respon.es to 50 msec l.rsts of repetitive cl ks of a susta ned un t (left colu n) an l a transient unit (right column) Each dot sl ows tl e occurrence of a nervous d sclarge The repetition rate of tl e clicks is ind cated by tl e inserted nun bers and tl e in l vid al cl cks are shown by tl e double dots below each recording seq ience

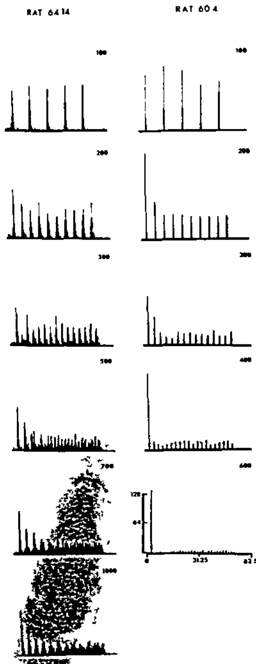


Fig 2 Post stimulus time histograms of responses to 50 msec bursts of repetitive clicks with various repetition rates As in Fig 1 the left column of recordings is from a sustained unit and the right column are recordings from a transient unit The click repetition rate is indicated by inserted legend numbers on each graph The scale in the lower right recording gives the time in msec on the horizontal axis and the number of spike counts are given on the vertical axis Each histogram represents the responses to 256 stimulus bursts

distribution of spikes in response to clicks of various repetition rate obtained from a sustained unit (left column) and that of a unit of the transient type (right column) The click repetition rate is given by legend numbers Similarities between the two types were noted when both were stimulated with clicks of low repetition rates whereas the responses differ markedly when higher click rates are used As seen there is a precise timing of the responses of the transient unit whereas the responses of the sustained unit fuses above a certain click repetition rate The majority of the responses of the transient unit falls within one channel of the analyser or is shared between two channels while the responses of the sustained unit is spread out over several channels

The "repetition rate selectivity" of four typical transient units is illustrated in Fig 3 A—D The stimulus consisted of repetitive clicks with various repetition rates delivered in bursts of 50 msec duration. The responses to the same type of stimulation of two sustained units is shown in Fig 3 E and F In all six graphs the average number of discharges during the stimulus duration was plotted as a function of click repetition rate The individual curves for each unit represent different sound intensities with 10 dB between each curve The dashed lines in Fig 3 A and B show what the response would be if each click evokes one discharge The discharge rate of all the transient units (A—D in Fig 3) is seen to depend strongly on the repetition rate of the stimulus clicks The firing rate of these units equalled the click rate up to a certain click rate Above this rate, (which varies among individual units) the discharge rate falls rapidly The rate of decrease in firing rate also varies somewhat in different units The figure also indicates that the "repetition rate selectivity" becomes less marked when the stimulus intensity is lowered The firing rate of the *sustained units however are but dependent on the click rate (Fig 3 E—F) and the* discharge rate only increases slightly with increasing click rate

The repetitions rate selectivity to clicks of the transient units could be equally well demonstrated by the use of continuous trains of clicks instead of clicks delivered in bursts The range within which each click is followed by a single discharge is however, smaller when using continuous trains of clicks than when the clicks are presented in 50 msec bursts The graphs in Fig 4 show the mean discharge frequency as a function of click frequency for stimulation with a 10 sec long train of clicks (open circles and for thirty two 50 msec long bursts of clicks (filled circles) In both cases the sound intensity was the same (*15 dB above threshold*)

No difference in response could be seen when the polarity of the electrical pulses applied to the condenser microphone was shifted in order to change the stimulus from compression to rarefaction clicks Nor was a change in the response pattern obtained when alternating click polarity was used

When the click stimulation was replaced by a series of short tone bursts delivered at a low repetition rate the transient units responded to each burst with a single discharge When repetition rate was increased or the duration of the individual bursts prolonged above a certain limit the firing ceases This selectivity is illustrated in Fig 5 A which shows the firing rate of a transient unit to short bursts of pure

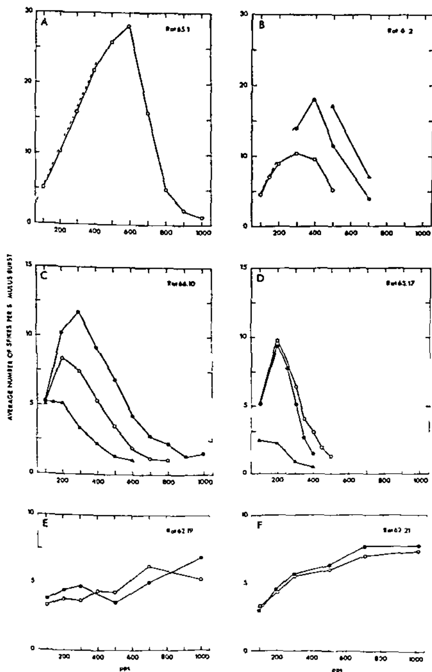


Fig 3 *A—D* Spike counts per stimulus burst as a function of repetition rate for 4 transient units The average number of discharges is shown as evoked by stimulation with thirty two 50 msec bursts of repetitive clicks The individual curves in B C and D show responses to clicks with different intensity (10 dB difference between each curve The straight dashed line in the two upper graphs shows the number of discharges which corresponds to one disc per click

E and F are similar plots of recordings from two sustained units

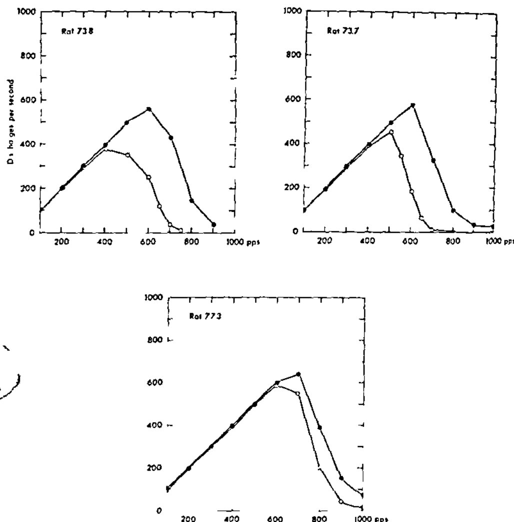


Fig. 4. Response of three transient units to clicks presented in 50 msec bursts (filled circles) and to a 10 sec burst (open circles). The average discharge frequency is shown as a function of the click repetition frequency.

tones presented repetitively as a function of repetition rate with the duration of the sound as parameter. The frequency of the tone was 15 kHz and the duration of the sounds are expressed by the number of full sinewaves which each burst consisted of (8, 16, 32 and 64 waves). The intensity of the sound was 15 dB above threshold.

When the duration of the sound was increased the range of repetition rate in which each sound evoked one discharge decreased. Fig. 5 B shows the average number of discharges which each toneburst evoked as a function of the reciprocal length of the silent period between the individual sound burst. For comparison the responses to click stimulation is also shown (filled circles) and connected with

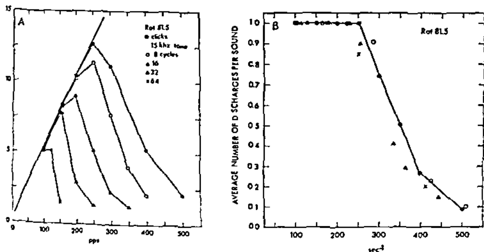


Fig 5 *A* Response to tone bursts of various durations compared to that of clicks The sounds were presented in 50 msec bursts in the same way as in Fig 4 and spike counts per burst are shown as a function of repetition rate

B The same data as in A but the average number of discharges per each sound is shown as a function of the reciprocal of the length of the silent period between the individual sounds The same symbols as in A are used The data points for click stimulation are connected by straight lines

straight lines The symbols indicating the durations of the sounds are the same in Fig 5 A and B It is apparent that the range in which the unit responded with one discharge depends on the length of silence between successive sounds and this range is almost independent of the repetition rate Thus these units are not selective to the repetition rate *per se* but rather to the duration of the silent period between successive stimulus sounds

The responses of the transient units to clicks can be completely inhibited by noise and by pure tones in certain frequency ranges More specifically pure tones with a frequency immediately below or above the best excitatory spectrum are usually most effective

Low frequency pure tones (below 1000 Hz) could not elicit any response in transient units nor could tones in which the amplitude was sinusoidally modulated at a frequency in the range in which click bursts elicited a response It should also be noted that the maximal firing rate of some of these units was more than 700 discharges per sec while the maximal firing rate of sustained units was never more than 400/sec

Discussion

The units in the rat which only respond to transient sounds are similar or identical to those described by Kiang (1965) and by Pfeiffer (1966) for the cochlear nucleus of the cat Those investigators however did not systematically study the respon

to transient sounds. Consequently they did not observe the specificity of these units to the repetition rate of transient sounds (or rather to the interval between individual sounds). Observations on the response pattern of rat transient units are valuable since they may elucidate the mechanisms underlying our ability to discriminate the pitch of certain sounds. It is an open question whether the ability to discriminate pitch is always due to spectral analysis like the one performed by the frequency selectivity of the basilar membrane (place theory) or if, under certain circumstances it is based on some time pattern analysis accomplished by the nervous system (volley theory). Numerous psychoacoustic investigations indicate that pitch discrimination of at least some sounds cannot be explained by spectral analysis. Thus for instance removal of the fundamental frequency of a train of repetitive clicks does not change the judged pitch of the sound (Schouten 1960). This procedure obviously removes the primary prerequisite for a pitch discrimination on a spectral basis, although the distance between individual harmonics still conveys information about the repetition rate of the clicks. Furthermore, recent psychoacoustic investigations have shown that the pitch of periodic clicks of a certain duration is under some circumstances, related to the duration of silence between these pulses rather than the periodicity of the pulses (McClellan and Small 1965, Rosenberg 1965, Zwislocki 1967). Although the duration of silence may have some weak spectral correlates other factors make it [illegible]lt to explain the phenomenon on the basis of spectral analysis similar to that [illegible] by the basilar membrane. Of additional interest to our findings is that this particular pitch phenomenon is only present at low repetition rates. The rat transient units display response patterns similar to the psychoacoustic finding regarding this particular type of pitch perception. The repetition rate selectivity of rat transient units is not a result of spectral analysis and shows that the discrimination is due to a time pattern analysis. This statement is supported by the fact that shifting the polarity of every second click in a train consisting of clicks of the same polarity did not change the response pattern. The precise timing of the transient units (Fig 1 and 2) makes it further plausible that these units play an active rôle in the perception of the periodicity of sounds.

There may be a widespread distribution of this type of units through the entire animal kingdom. Roeder (1966) has found similar units located in the mesothoracic ganglion of certain nocturnal moths. He found that some of these "pulse marker" units only respond once for each stimulus and that they needed a certain period of silence before a new response could be evoked. In this context it should be mentioned that in the somatic sensory cortex of cats Mountcastle, Davies and Berman (1957) found a similar type of unit. They called these unit "cut-off" units since they followed the repetitive peripheral stimulation up to a certain rate above which they ceased to respond.

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The Secretion of Adrenaline and Noradrenaline in 20° C and 3° C Acclimated Rats Injured by Limb Ischemia

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Abstract

Stoner H B and B Westerholm *The secretion of adrenaline and noradrenaline in 20° C and 3° C acclimated rats injured by limb ischemia* Acta physiol scand 1969 *75* 552—564

The effects of hind limb ischemia on the concentrations of adrenaline and noradrenaline in the adrenals heart and spleen on the excretion of these compounds and their 3 0 methylated metabolites in the urine and on the uptake of (±) noradrenaline 7 ^{3}H by the adrenals kidneys een and heart have been studied in 20° C and 3° C acclimated rats at an environmental perature of 20° C The adrenals of uninjured 3° C acclimated rats contained more aline and noradrenaline than those from 20° C acclimated rats In a 20° C environment oth types of rat excreted the same amounts of these compounds Hind limb ischemia increased e rate of secretion of adrenaline and noradrenaline since the adrenal contents of these compounds were reduced their excretion and that of two of their metabolites was increased and tissue uptake was not reduced The response was the same in 20° C and 3° C acclimated rats after a 4 hr period of bilateral hind limb ischemia According to the assay methods used adrenaline secretion preceded that of noradrenaline and the secretion of both continued for up to 26 hrs after the injury The possible factors in the injury leading to the increase in catecholamine secretion are discussed and also the relation between these changes and some of the biochemical sequelae of limb ischemia

It is generally agreed that noxious stimuli activate the sympathetic adrenal system leading to the secretion of adrenaline and noradrenaline These catecholamines play a causal role in many of the subsequent biochemical changes particularly in the increases in the concentrations of glucose and non esterified fatty acid in the plasma Injury to the body such as that produced by hemorrhage and damage to tissues *must* also be expected to stimulate this system

In man surgical operations increase the urinary excretion of catecholamines which reaches a maximum after about two days and returns to normal by the fourth to seventh day (Pekkarinen 1960) In burned patients particularly the increased output of catecholamines can continue for a surprisingly long time (Birke *et al* 1958) Numerous factors may be involved in the response of such patients and experimental work is necessary to define them Most of the experimental work, re

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viewed by Chien (1967), has been done on anesthetized dogs where oligemic hypotension has been shown to increase the circulating levels of adrenaline and noradrenaline. The biochemical response to injury has probably been most studied in the rat where there is less direct information on the catecholamine changes although Young and Gray (1956) have shown that plasma catecholamine concentrations increase in the rat after tumbling trauma

Hemorrhage and tumbling trauma are essentially acute injuries and it was of interest to study the effect of a more prolonged injury such as that produced by limb ischemia The object was to determine the time relations between the secretion of adrenaline and noradrenaline and the injury, to define the stimuli for catecholamine secretion and to relate the catecholamine secretion to the biochemical response to the injury Limb ischemia was produced by rubber tourniquets which require only a short ether anesthesia for their application Both 20° C and 3° C acclimated rats were used Provided that the latter have not been kept too long in the cold, the size of the injury produced by hind limb ischemia and its mortality rate is the same as in the 20° C acclimated rats but the response to the injury differs in certain respects (Stoner 1965) A major difference is the better preservation of heat production after injury with a slower fall in core temperature This is related to the altered activity of the sympathetic nervous system in the cold acclimated rat (Stoner 1965) and the question arises whether it is due to greater secretion of catecholamines after injury or to their increased sensitivity to catecholamines (Hsieh and Carlson 1957 Depocas 1960 Heroux 1961)

Methods

Male albino W [illegible] M R C diet 41 B (Bruce and Parkes 1956) wer [illegible] C acclimated rats were kept from wea [illegible] The 3° C acclimated rats had been kep [illegible] temperature of 2—4° C for 16—21 day [illegible] (For definition of acclimation see Hart 1961)

All the tests were carried out at an environmental temperature of 18—22° C The air temperature did not vary more than 0 5° C during any one experiment Bilateral hind limb ischemia was produced by Rosenthal s (1943) method with rubber tourniquets applied between 9 and 11 a.m under ether anesthesia The uninjured controls were given 3 min ether anesthesia at [illegible] and water was available when the experiment lasted overnight

The rats were decapitated and the ventricle spleen adrenals and kidneys rapidly excised and dropped into liquid nitrogen They were then weighed and homogenized in 10 0 ml ice cold 0 4 N perchloric acid Adrenaline and noradrenaline in the urine and tissues were extracted and determined spectrophotofluorimetrically by the method of Haggendal 1963 Metanephrine and normetanephrine in urine were extracted by the method of Carlsson and Lindqvist (1962 and determined by the method of Haggendal (196 No attempt was made to measure the amounts of conjugated catecholamines in the urine

In the experiments on the uptake of noradrenaline by the tissues + noradrenaline 7 (8 C/mmole New England Nuclear Corp Boston U S A was used Before use th diluted to 100 μC per ml with 0 9 per cent NaCl containing 0 005 per cent ascorbic rat was given 100 μC per kg body wt by injection into a tail vein The anim capitated 5 min later The blood was collected in heparinized beakers and the

rinsed in 0 9 per cent NaCl, blotted free of adhering liquid and dropped into liquid nitrogen The blood (0 5—1 0 ml) and organs were extracted with 5 0 ml 0 4 N perchloric acid (Sjöqvist, Taylor and Titus 1967) After centrifugation 0 25 ml of the supernatant was transferred to a counting vial containing 12 ml Bray's (1960) solution and the radioactivity assayed in a Packard Tri-carb scintillation spectrometer using ^{3}H toluene (Packard Instrument Co, La Grange, Illinois U S A) as an internal standard The plasma volume was calculated using the haematocrit value as described by Ashby, Heath and Stoner (1965) In both types of rat the average value 1—3 hrs after removal of the tourniquets was 59

The results have been expressed as geometric means with the standard error range for the reasons given by Heath (1967) The means were compared statistically by Student's '*t*' test

Results

Of the 40 rats in these experiments which were observed for 26 hrs after 4 hrs bilateral hind-limb ischemia only one died The rest were seriously ill at this time but it seemed likely that they would recover All showed the typical signs of shock in the rat (Stoner 1961) The rats subjected to 2 hrs bilateral hind-limb ischemia were less severely affected and none died

Tissue Concentrations of Adrenaline and Noradrenaline

In the *heart* the concentrations of adrenaline [0 07(0 05—0 11)μg/g] and noradrenaline [0 34(0 30—0 38)μg/g] were in the range found by other authors (*e g* Johnson 1966, Gutman and Weil-Malherbe 1967) These concentrations were not [illegible] dly affected by acclimation to 3° C nor by 4 hrs limb ischemia samples [illegible]ng taken up to 2 hrs after removal of the tourniquets

The amounts in the *spleen* (Table I) are expressed as μg per kg body wt since the weight of the spleen varied considerably with differences in its content of blood In these experiments the first specimens *i e* those from the controls at zero time and from the injured 4 hrs before removal of the tourniquets in Table I, were taken immediately after the application of the tourniquets or during the 3 min period of ether anesthesia in the controls The adrenaline content of the spleen was not affected either by injury or by cold-acclimation The noradrenaline content of the spleen was higher at the beginning of the experiment than in the later stages in both controls and injured Comparison with the results of Iversen Glowinski and Axelrod (1966) and Johnson (1966) indicated that the later values were within the normal range The high initial values could be due to the anesthetic since there was no difference in this respect between controls and injured Cold acclimation did not affect the noradrenaline content of the spleen and there were no significant differences between the injured and their controls at any of the times studied

The *adrenal* contents of both adrenaline and noradrenaline are shown in Table II The control values did not vary significantly during the day and they have all been grouped together The values for both catecholamines were significantly higher in the 3° C acclimated control rats The percentage increase was about the same for each compound In the injured rats no changes occurred during the 4 hr period of limb ischemia but when the tourniquets were removed there was a progressive fall in the contents of both adrenaline and noradrenaline The most striking change

TABLE I Adrenaline and noradrenaline contents of the spleen expressed as $\mu g/kg/b$ w in control and injured rats The latter were subjected to 4 hr bilateral hind limb ischemia Results expressed as geom mean $\pm$ standard error range Four rats in each group environmental temperature 18—22° C

Control	Time (hrs) after ether	0†	4	5	6
20° C acclimated	Adrenaline	0.09	0.14	0.18	0.17
		(0.04—0.18)	(0.08—0.27)	(0.09—0.37)	(0.09—0.33)
	Noradrenaline	2.55	1.08*	0.84	1.57
		(2.13—3.05)	(0.92—1.28)	(0.45—1.59)	(1.31—1.88)
3° C acclimated	Adrenaline	0.14	0.10	0.26	0.19
		(0.07—0.27)	(0.05—0.23)	(0.14—0.48)	(0.10—0.39)
	Noradrenaline	3.05	1.48**	1.22	1.91
		(2.67—3.50)	(1.33—1.65)	(0.79—1.90)	(1.66—2.19)
Injured	Time (hrs) after removal of torniquets	—4†	0	1	2
20° C acclimated	Adrenaline	0.36	0.20	0.13	0.21
		(0.26—0.43)	(0.15—0.28)	(0.07—0.24)	(0.10—0.44)
	Noradrenaline	2.13	1.10**	1.03	1.61
		(1.99—2.29)	(0.96—1.27)	(0.58—1.83)	(1.37—1.88)
3° C acclimated	Adrenaline	0.08	0.28	0.19	0.21
		(0.05—0.13)	(0.24—0.34)	(0.10—0.39)	(0.10—0.43)
	Noradrenaline	3.82	0.63**	1.12*	1.68*
		(3.30—4.41)	(0.41—0.98)	(0.74—1.70)	(1.39—2.03)

No significant difference between values in injured and controls
In comparisons with initial values — *difference significant at $P < 0.05$ **difference significant at $P < 0.01$ †see text

occurred in the adrenaline fraction so that there was an increase in the percentage of the combined content formed by noradrenaline

A 2 hr period of bilateral hind limb ischemia had less effect In five 20° C acclimated rats killed 26 hrs after this injury the adrenaline content of the adrenals was 60.9 (51.9—71.4) μg per kg body wt significantly ($P<0.05$) less than in the controls but obviously more than in those injured by the longer period of limb ischemia The noradrenaline content of these adrenals [17.2 (12.4—23.8) μg per kg body wt] was not significantly different from the control value

Urinary Excretion of Adrenaline and Noradrenaline and their 3 O Methylated Metabolites

Under our experimental conditions in a 20° C environment there was no differe in the 24 hr excretion of adrenaline and nor adrenaline by the 20° C and

TABLE II Effect of 4 hr period of bilateral hind limb ischemia on the adrenaline and noradrenaline contents of the adrenals of 20° C and 3° C acclimated rats Results expressed in μg kg b w and given as the geom mean ± standard error range Number of rats given in italics Environmental temperature 18—22° C

		Controls	End of limb ischemia	1—2 hrs after	26 hrs after
20° C acclimated	Adrenaline	87 6	92 7	66 4	22 8***
		(82 6—92 9)	(84 7—99 9)	(56 2—78 4)	(17 5—29 7)
	Noradrenaline	21 6	25 4	32 8*	10 4*
		(19 7—23 7)	(21 1—30 7)	(28 9—37 3)	(7 5—14 4)
		26	*4*	*8*	*9*
3° C acclimated	Adrenaline	112 7***	99 1	54 2*	11 2**
		(105 2—120 8)	(87 3—112 4)	(40 9—71 8)	(6 0—20 5)
	Noradrenaline	29 4*	28 6	32 1	7 8**
		(26 8—32 4)	(23 5—34 7)	(28 1—36 8)	(5 2—11 6)
		21	*4*	*8*	*5*

3° C acclimated control values compared with 20° C acclimated control values
Values in injured rats compared with appropriate controls

* difference significant at P 0 05
** „ „ „ P 0 01
*** „ „ „ P 0 001

TABLE III Amounts of adrenaline and noradrenaline excreted by 20° and 3° C acclimated rats during the 26 hrs following 2 and 4 hr periods of bilateral hind limb ischemia and by uninjured controls during the same period Environmental temperature 18—22° C

Acclimation temp C	Group	Number of rats	Adrenaline μg kg b w mean ± S E range	Noradrenaline
20	Control	10	2 62 (2 44—2 82)	5 01 (4 44—3 73)
20	2 hr ischemia	4	5 53 (4 89—6 26)***	14 01 (11 53—17 64)*
20	4 hr ischemia	9	6 55 (5 22—8 23)**	9 44 (8 19—10 [illegible])**
3	Control	5	2 58 (2 34—2 86)	5 39 (4 93—5 90)
3	4 hr ischemia	5	10 96 (9 54—12 60)***	8 75 (7 67—9 98)*

*** difference from controls significant at P 0 001
** „ „ „ „ „ P 0 01
* „ „ „ „ „ P 0 02

No significant differences between the values after 2 and 4 hr ischemia in 20° C acclimated rats or between the values after 4 hr ischemia in the 20° and 3° C acclimated rats

TABLE IV The excretion of adrenaline and noradrenaline in the urine, expressed as μg per kg b w per hr by control 20° C acclimated rats and 20° C acclimated rats subjected to bilateral hind limb ischemia from 2—4 hrs Results expressed as geom mean ± standard error range Five rats in each group Environmental temp 18—22° C

Time (hrs) from start of experiment		0—4 hrs	4—8 hrs	8—12 hrs	12—24 hrs	24—30 hrs
Group	*Compound*					
Control	Adrenaline	0 12 (0 11—0 13)	0 05 (0 04—0 08)	0 14 (0 13—0 15)	0 12 (0 11—0 14)	0 08 (0 07—0 10)
	Nor-adrenaline	0 17 (0 13—0 22)	0 10 (0 08—0 12)	0 22 (0 20—0 24)	0 07 (0 05—0 11)	0 25 (0 22—0 28)
2 hr ischemia	Adrenaline	0 17* (0 15—0 19)	0 07 (0 04—0 12)	0 51** (0 45—0 58)	0 20* (0 17—0 24)	0 09 (0 07—0 12)
	Nor-adrenaline	0 19 (0 15—0 23)	0 15 (0 11—0 21)	0 86** (0 73—0 98)	0 31** (0 27—0 36)	0 87** (0 63—1 18)

* difference significant at P < 0 05 ** difference significant at P < 0 01

Rate in the injured compared with the rate in the controls for the same period

acclimated control rats The former excreted 2 75 (2 55—2 96) μg adrenaline and 4 56 (3 91—5 33) μg noradrenaline per kg body wt and the latter excreted 2 57 (2 36—3 01) μg and 4 89(4 25—5 62) μg respectively The variations in the excretion rates in the controls during the 24 hrs (Table IV and V) may reflect their normal metabolic and activity rhythms

In both 20° C and 3° C acclimated rats adrenaline excretion was increased during the period of ischemia (Table IV and V) For the rats given 2 hr bilateral hind limb ischemia this increase was underestimated since urine was collected for 2 hrs before the tourniquets were applied Noradrenaline excretion was unaltered during the period of limb ischemia

The total amounts of adrenaline and noradrenaline excreted during the 26 hrs following removal of the tourniquets are shown in Table III The amounts of both were increased but were not significantly different after the 2 and 4 hr injuries in the 20° C acclimated rats nor was there any difference between the effects of the 4 hr injury in the 20° and 3° C acclimated rats Some idea of the relationship between the injury and catecholamine secretion was derived from the study of shorter collection periods

After a 2 hr period of bilateral hind limb ischemia in 20 C acclimated rats (Table IV) the rate of adrenaline secretion rose further reaching its maximum after 4 to 8 hrs and then falling to a normal level after 20 hrs Noradrenaline excretion rose similarly but remained at a high level for much longer

TABLE V The excretion of adrenaline and noradrenaline in the urine, expressed as μg per kg b w per hr, by control rats and rats subjected to bilateral hind limb ischemia from 0—4 hr Results expressed as geom mean ± standard error range Number of rats shown in italics *Environmental temp 18—22° C*

Collection period Acclimation temp and group	Compound	0—4 hrs	4—8 hrs	8—12 hrs	12—30 hrs
20° C Control	Adrenaline	0 13	0 06*	0 12	0 10
		(0 11—0 16)	(0 04—0 08)	(0 10—0 13)	(0 09—0 11)
	Noradrenaline	0 23	0 06**	0 29	0 17
		(0 20—0 26)	(0 04—0 09)	(0 25—0 33)	(0 14—0 19)
		15	*9*	*10*	*10*
20° C Injured	Adrenaline	0 31**	0 20*	0 28*	0 26***
		(0 26—0 36)	(0 12—0 34)	(0 20—0 38)	(0 24—0 29)
	Noradrenaline	0 17	0 13	0 25	0 33**
		(0 11—0 24)	(0 08—0 22)	(0 15—0 43)	(0 29—0 38)
		14	*9*	*9*	*9*
3° C Control	Adrenaline	0 09	0 17	0 02	0 05
		(0 07—0 12)	(0 14—0 19)	(0 006—0 04)	(0 03—0 10)
	Noradrenaline	0 08	0 21*	0 22*	0 18
		(0 05—0 12)	(0 18—0 26)	(0 18—0 26)	(0 16—0 20)
		14	*4*	*4*	*5*
3° C Injured	Adrenaline	0 33**	0 60**	0 12	0 30*
		(0 27—0 40)	(0 50—0 73)	(0 04—0 39)	(0 28—0 33)
	Noradrenaline	0 16	0 20	0 08	0 36*
		(0 14—0 19)	(0 14—0 27)	(0 03—0 21)	(0 33—0 41)
		15	5	5	5

* difference significant at P 0 05
** " " " P < 0 01
*** " " " P 0 001

For Controls comparison made with rate of excretion during first 4 hrs For Injured comparison made with the rate in the Controls for the same period

After 4 hrs ischemia in both 20 and 3° C acclimated rats (Table V) the increased excretion of adrenaline which had begun during the ischemic period continued for a much longer time and was still present during the 8—26 hr period after removal of the tourniquets Noradrenaline excretion in these rats increased much more slowly and was only significantly higher than in the controls between 8 and 26 hrs after removal of the tourniquets There were no significant differences between the responses of the 20° and 3° C acclimated rats

The 24 hr excretion of metanephrine and normetanephrine was greater in the injured rats than in their controls the difference being most marked in the 20° C acclimated rats (Table VI)

Uptake of (±)-Noradrenaline 7 ³H

Most of the noradrenaline secreted into the blood is taken up into other adrenergic

TABLE VI 24 hr excretion (μg per kg/b w) of free metanephrine and normetanephrine in 20 C and 3° C acclimated control and injured rats 24 hrs measured from the beginning of the 4 hr period of bilateral hind limb ischemia or from the control period of ether anesthesia. Results expressed as geom. mean ± standard error range Environmental temp. 18—22° C.

	Control	Injured	Control *vs* Injured
20° C acclimated			
metanephrine	1 8	7 3	P < 0 001
	(1 7—2 0)	(5 9— 9 1)	
normetanephrine	4 8	13.2	P < 0 01
	(4 0—5 8)	(10 8—16 1)	
Number of rats	5	6	
3° C acclimated			
metanephrine	0 9*	2 9	P < 0 05
	(0 7—1.2)	(2 1— 4 0)	
normetanephrine	6 6	8 7	—
	(5 9—7 4)	(8 3— 9 2)	
Number of rats	4	5	

* significantly less than 20° C acclimated controls, P < 0 05

nerve-endings The effect of injury on this major pathway for the removal of circulating noradrenaline must be examined before interpreting the above results (±) Noradrenaline 7-³H was injected i v and the blood and tissues examined 5 min later The choice of this interval was based on the work of Whitby Axelrod and Weil Malherbe (1961) and on the experiments of Pareira Serkes and Lang (1960) on mixing in the blood compartment of injured rats

Five minutes after the injection of (±) noradrenaline 7 ³H the same fraction of the dose remained in the plasma in the controls and in the experimental rats at the end of the period of ischemia and 2 hrs after removal of the tourniquets irrespective of the temperature of acclimation (Table VII) In addition to the tissues shown in that table uptake by the adrenals was also examined and found to be less than 0 1 per cent in all groups Injury did not affect the uptake by the ventricle or spleen. The only significant difference between the control and injured rats was the increased uptake by the kidney 2 hrs after removal of the tourniquets in both 20° C and 3° C acclimated rats (Table VII

Discussion

While a 2 hr period of bilateral hind limb ischemia at an environmental temperature of 20° C is never fatal in our rats a 4 hr period has usually given a much higher mortality rate (about 80 %) than that found in these experiments This difference from our usual finding with unconfined rats is attributed to the use of restraining cages throughout the experiment. Although the cages used do not cause 'rest

TABLE VII Tissue uptake of (±) noradrenaline 7 ³H 5 min after its i v injection in control and injured rats Injured rats tested at the end of 4 hr period of bilateral hind limb ischemia and 2 hrs later Number of rats in each group shown in italics Environmental temp 18—22° C

Group	Control	End of limb limb ischemia	2 hrs after limb ischemia	Control	End of limb ischemia	2 hrs after limb ischemia
Acclimation temp	20° C			3° C		
Per cent of dose in plasma compartment	2 44 (2 04—2 91) *9*	3 09 (2 65—3 60) *6*	2 84 (2 69—3 00) *5*	2 67 (2 37—3 01) *9*	2 66 (2 11—3 35) *4*	2 73 (2 28—3 28 *4*
Uptake Per cent of dose in						
Kidneys	1 58 (1 32—1 90) *9*	1 46 (1 39—1 54) *6*	3 75* (3 12—4 51) *5*	1 78 (1 60—1 98) *9*	1 84 (1 76—1 92) *4*	3 58** (3 22—3 99 *4*
Spleen	0 22 (0 18—0 27) *6*	0 26 (0 21—0 32) *6*	0 18 (0 15—0 21) *5*	0 22 (0 19—0 26) *9*	0 22 (0 17—0 29) *4*	0 13 (0 10—0 18 *4*
[illegible]tricle (g wet wt)	2 35 (2 10—1 64) *9*	2 90 (2 57—3 27) *4*	3 05 (2 93—3 18) *5*	2 11 (1 92—2 31) *9*	2 15 (1 87—2 47) *4*	2 35 (1 98—2 79 *4*

Values expressed as geom mean – standard error range

* significantly different from control at P 0 01

** " " " " " P 0 001

hypothermia they do affect some responses to injury (Stoner and Little 1968*b*) The lower mortality rate is possibly due to a slower rate of fluid loss into the damaged tissue as a result of the altered posture of the hind limbs Nevertheless the condition of the rats showed that this was a severe injury with prolonged effects This was true for both 20° C and 3 C acclimated rats It must be emphasized that in the cold acclimated rats the period of exposure to 3 C before the experiment was insufficient to alter the proportional weight of the hind quarters so that the amount of tissue injured by the ischemia would be the same as in the 20 C acclimated rats (Stoner 1965)

Adrenaline and noradrenaline concentrations in the tissues and urine of the uninjured 20° C acclimated rats were in agreement with those reported by Leduc (1961) Le Blanc and Nadeau (1961 Smith and Dugal (1964*a* Iversen *et al* (1966) Graham (1966) and Johnson 1966 Comparison with Graham s results is particularly useful since he found that keeping rats for 12 hrs without food or water in a restraining cage led to a marked increase in the urinary excretion of adrenaline and noradrenaline As this did not occur in our control rats confined for 30 hrs our

experimental arrangements with food and water available would not appear to impose a very severe stress

The most detailed study of catecholamine metabolism in cold acclimation is by Leduc (1961) He found an increase in the adrenaline, but not in the noradrenaline, content of the adrenals and an increase in the urinary excretion of both adrenaline and noradrenaline in rats continuously exposed to 3° C These results have been confirmed by Le Blanc and Nadeau (1961), Smith and Dugal (1964*a*), Bertin and Chevillard (1967) and Chevillard and Bertin (1967) The last named authors also reported an increase in the noradrenaline content of the adrenals under these conditions It is difficult to compare our results directly as, apart from differences in age and strain, our cold acclimated rats had always been removed from the cold to a 20° C environment before sampling Under these conditions both the adrenaline and noradrenaline contents of the adrenals of the cold acclimated rats were significantly increased but the urinary excretion of these compounds was not different from 20° C acclimated rats at this environmental temperature It would appear that the environmental stress in the two groups was the same and the secretion of catecholamines was very low and similar Smith and Dugal (1964*a*) have shown that the increased catecholamine excretion of the rat in the cold rapidly falls when the environmental temperature is raised from 2° C to 23° C The similarity between the adrenaline and noradrenaline contents of the ventricle and spleen in 20° C and 3° C acclimated rats was to be expected from the work of Leduc (1961) bearing in mind the length of time our rats had been exposed to cold

The assessment of sympathetic adrenal medullary activity cannot rest on the measurement of a single parameter Secreted catecholamine is removed in three ways, by uptake into adrenergic tissues the most important pathway (Gillespie 1966) by metabolism and by excretion of the unchanged compounds and their metabolites Although <5 % is excreted unchanged in the urine the assay of the compounds in the urine is important because it gives a continuous measure of the blood level Leduc (1961) found that the percentage of injected doses of adrenaline or noradrenaline excreted unchanged in the urine was not altered by cold acclimation These findings have been questioned by Smith and Dugal (1964*b*) who reported an increase in the percentage excretion by cold acclimated rats However the difference would not be large enough to affect the interpretation of our results

In our experiments hind limb ischemia increased the rate of secretion of adrenaline and noradrenaline since the adrenal contents of these compounds were reduced their excretion and that of two of their metabolites was increased and tissue uptake was not reduced

In the 20° C acclimated rats while the changes in the adrenals were proportional to the severity of the injury those in the urine were not The similar changes in the urine after 2 and 4 hr periods of bilateral hind limb ischemia were unexpected and difficult to explain particularly in the case of adrenaline where proportionality between adrenaline mediated effects such as hyperglycemia and the size of the injury is well established Renal function is better preserved after the smaller injury

these circumstances the urine concentrations may reflect those in the blood more accurately The increased uptake of tritiated noradrenaline by the kidneys 2 hr after removal of the tourniquets may reflect increased sympathetic discharge in these kidneys with consequent disturbance in renal hemodynamics

In the 3° C acclimated rats subjected to 4 hr bilateral hind limb ischemia in a 20° C environment the size of the injury and the environmental stress was the same as in the 20° C acclimated rats treated in this way (see above) Under these conditions the response to the injury appeared to be very similar in the two types of rat judging by the changes in the adrenals and urine This implies that the better maintenance of body temperature and O_2 consumption by the injured cold acclimated rat although catecholamine dependent, is related to their greater sensitivity to these compounds and not to increased secretion (Hast 1960, Stoner 1965 Stoner and Little 1968*a*)

As in man after surgery (Coward and Smith 1966) we found an increase in the excretion of metanephrine and normetanephrine in the injured rats Although it was possible to distinguish between these metabolites the analysis is difficult and we would not want to attach any importance to apparent differences in the responses of the 20° and 3° C acclimated rats

No changes were found in the adrenaline or noradrenaline contents of the spleen r ventricle after limb ischemia although decreases have been reported after orrhage (Chien 1967) This was probably because the injury although severe d not fatal and the organs were sampled in the early stages of the response before impairment of oxygen transport

As in the clinical work referred to in the Introduction the increased secretion of catecholamines continued for a considerable time after limb ischemia in fact into the recovery period There would also appear to be time differences in the relation of the adrenaline and noradrenaline responses to the injury Judging by the changes in urinary excretion the increase in adrenaline secretion occurred before that in noradrenaline This was borne out to some extent by the adrenal changes although these lagged far behind those in the urine A similar sequence was described by Millar and Benfey (1958) after hemorrhage in the dog It must however be kept in mind that both these compounds have great biological activity Significant amounts of both could have been secreted which would not be detectable by the assay methods used

These considerations are particularly important in comparing the timing of these responses with those biochemical sequelae of injury which are known to be dependent on adrenal sympathetic activity The breakdown of glycogen in the uninjured muscle of the injured rat prevented by adrenal medullectomy (Stoner 1958 begins with the application of the tourniquets and continues throughout the experiment This is in keeping with the changes in the urinary excretion of adrenaline Mobilisation of triglyceride after hind limb ischemia was not prevented by adrenal medullectomy (Stoner 1962) and is best indicated by the degree of lipolysis in the fat depots (Stoner and Matthews 1967) The nonesterified fatty acid content of the epididymal fat pads was high from the time of application of the tourniquets This evidence of

increased adrenergic activity was not mirrored in any of the parameters studied here. It may be that these biochemical changes in the fat and to a lesser extent in the plasma (Stoner and Matthews, 1967) are the most delicate indicators of such activity in the rat. Leduc (1961) concluded that noradrenaline secretion was the first line of defence to cold stress and that adrenaline secretion was the second. There is insufficient evidence here to say that the position is reversed after injury.

In the early part of the experiment the reflex increase in catecholamine secretion was probably due to afferent impulses from the damaged area. Since the injury interfered with the innervation of the hind limbs these stimuli would decrease and be replaced after removal of the tourniquets, by those due to the ensuing hypovolemia. The swelling of the injured limbs is maximal after about 2 hrs (Haist and Hamilton 1944) when the hematocrit values indicated about a 50 % reduction in the plasma volume of both 20° C and 3° C acclimated rats injured by 4 hr bilateral hind limb ischemia. Walker *et al* (1959) considered a fall in blood volume to be a more important stimulus for adrenal medullary secretion than hypotension. Our results would support this view since a 2 hr period of bilateral hind limb ischemia does not lower the blood pressure (Dexter, Stoner and Green 1953) and the hypotension after a 4 hr period is neither severe nor progressive in non fatal cases (Stoner and Little 1968*b*).

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Effect of Reserpine on Accommodation and Pupil Diameter in Monkeys (Macaca irus)

By

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Abstract

Tornqvist, G *Effect of reserpine on accommodation and pupil diameter in monkeys (Macaca irus)* Acta physiol scand 1969 75 565—567

Pupil diameter and refractive state were measured in cynomolgus monkeys under pentobarbital before and after i m administration of reserpine 2 and 5 mg/kg Reserpine was found to cause only a small miotic response and no accommodation at all

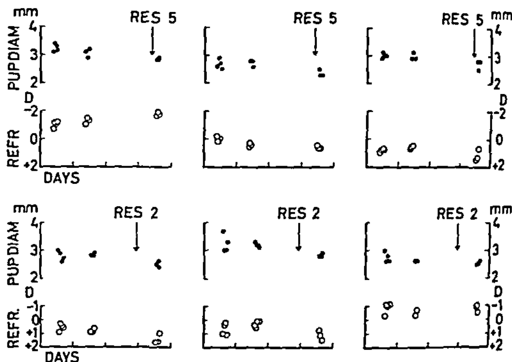
One marked effect of reserpine in many experimental animals is the distinct miosis (Tripod *et al* 1953, Bein *et al* 1953) In primates and humans the miosis is less distinct and not necessarily seen in all individuals (Bein 1956) The miosis is thought to be central in origin (Bein 1956) This assumption and the finding by Dahlstrom *et al* (1964) of a richly developed adrenergic synaptic system in the Edinger-Westphal nucleus of the rat initiated the present experiments Does reserpine induce accommodation by a central mechanism? If this were so it would be important for the study of the pharmacology and physiology of accommodation and of aqueous humor dynamics Monkeys were chosen as experimental animals because, in contrast to rodents, they have a large accommodation amplitude

Material and methods

Six cynomolgus monkeys weighing 2 5—3 8 kg and of both sexes were used The monkeys were all young adults and known to be able to accommodate more than 10 diopters (D The [illegible] d elsewhere [illegible] rforme [illegible] wh [illegible] constant illumination The refraction measurements were performed on the [illegible] The measurements were done by means of a Thorner (1927) optometer This [illegible] the spectacle plane refraction in diopters (D)

On the first and second day of an experiment prevalues of the pupil diameter [illegible] state were read during pentobarbital anesthesia. Pupil and refraction were measur[illegible]

* Present address of author



g 1 Effect of i m reserpine on pupil diameter (solid circles) and refractive state (open les)

Every solid circle represents the mean of 4 single readings of the pupil diameter and every open circle represents the mean of 10 individual refraction readings Such mean values were obtained for both pupil and refraction once every hour for 3—4 hrs Three monkeys were given 5 mg/kg of reserpine (top row) and 3 others 2 mg/kg

3—4 hrs Every pupil measurement was taken as the mean of four and every refraction value as the mean of 10 single readings The mean values, one every hour, are the values plotted in Fig 1

The reserpine was given i m in the evening of the third day (3 animals, 2 mg per kg) or in the morning of the fourth day (3 animals 5 mg per kg) On the fourth day the animals were again anesthetized and measurements of the pupil and refractive state were taken as just described

The reserpine (Serpasil Ciba was given i m in a concentration of 2 5 mg per ml and in a dose of 2 or 5 mg per kg The 2 mg per kg dose was given 15 hrs, and the 5 mg dose 3 hrs [illegible] fourth day The reliability of the [illegible] 1967) The standard error of the [illegible] ess than 0 1 mm and the standard [illegible] was less than 0 1 D

It has been found that an accommodative response cannot be elicited when the blood pressure is very low Therefore in 3 monkeys the blood pressures were recorded with an intra femoral catheter and a pressure transducer One of the monkeys was given 2 and the two others 5 mg per kg of reserpine The mean arterial pressures were found to be well above the critical level (about 60—70 mm Hg) influencing accommodation

Results

The most conspicuous result seen after reserpine administration to the monkeys was their loss of aggressivity In agreement with the literature the monkeys became docile and were easy to handle Undoubtedly the reserpine had been absorbed

The effect on pupil and refractive state are shown in Fig 1 No dramatic effects

are seen neither 3—5 hrs after 5 mg per kg reserpine nor 15—17 hrs after 2 mg per kg It is seen that all 6 monkeys showed decrease in pupil diameter after reserpine but the change was very small 0 2—0 5 mm

The effect on refractive state was more variable One monkey receiving 5 mg per kg showed a small change towards myopia, one other receiving 2 mg per kg a small hyperopic change In 4 monkeys no obvious changes in refraction took place Thus the effects on refraction were insignificant and obviously no accommodation could be induced

Discussion

The main purpose of the present investigation was to see if it is possible to induce accommodation by reserpine, but the observations of pupil size are also of interest The drug gives miosis in many animals by a presumed central mechanism (Bein 1956) Dahlstrom *et al* (1964) found catecholamine accumulating fibres in the Edinger Westphal nucleus and suggested that there is an adrenergic, inhibitory tone to the third nerve neurons This inhibitory tone probably is responsible for the miosis caused by reserpine However, in agreement with earlier reports the present experiments demonstrate that in primates reserpine gives only a small pupillary constriction Besides the pupillary parasympathetic centre the Edinger Westphal nucleus is also considered to contain the centre of accommodation If there is an adrenergic inhibitory mechanism to this centre reserpine would likely induce accommodation Accommodation cannot be studied in rats (used by Dahlstrom *et al*) as they have practically no accommodative ability However reserpine gives no accommodation in monkeys either (though these animals have a large accommodation amplitude)

It can be argued that in monkeys during barbiturate sleep there is perhaps no parasympathetic activity in the central neurons regulating accommodation In such a case the presumed inhibitory adrenergic terminals could not act and reserpine would have no effect However, there is evidence that an activity exists in the Edinger Westphal nucleus of pentobarbital anesthetized monkeys physostigmine elicits accommodation and moreover after systemically given hexamethonium there is a distinct relaxation of accommodation (Tornqvist 1967)

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Intercostal-to-Phrenic Reflexes in the Spinal Cat

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Abstract

DECIMA, E E, C v EULER and U THODEN *Intercostal to phrenic reflexes in the spinal cat* Acta physiol scand 1969 75 568—579

The diaphragm, which previously has been shown to contain relatively few proprioceptors and to lack autogenetic facilitation was found to be under reflex control from intercostal afferents Electrical stimulation of the intercostal nerves of the lowest thoracic segments elicited a polysynaptic reflex excitation of phrenic motoneurones followed by a depression of spontaneous phrenic motor activity No phrenic responses were elicited from the upper thoracic segments Increased phrenic activity was also elicited by mechanical stimulation of the trunk In the receptive area of these effects was similarly limited to the region of the insertion of the diaphragm Alike reflex effects were obtained from both the internal and the external intercostal nerves In the internal nerve both group Ib and II afferents were found to contribute to the reflex whereas in the external nerve it was mainly the secondary muscle spindle endings which gave rise to the response In contrast to the intercostal to intercostal reflexes cutaneous afferents proved to be without effect in the intercostal to-phrenic reflex

The diaphragm differs from most skeletal muscles including the intercostal musculature, in that it has only a scanty supply of proprioceptors (Glebovskii 1962 Yasargil 1962, Corda, Euler and Lennerstrand 1965) among the few present the proportion of muscle spindles to tendon organs is low (*cf* Corda *et al* 1965*a*) In harmony with this all attempts to demonstrate autogenetic facilitation of the diaphragm have failed so far, while autogenetic inhibition is readily exhibited In contrast autogenetic facilitation has ben found to be a dominant feature of the motor control of the main synergist the external intercostal muscle This discrepancy in proprioceptive control between the two sets of respiratory muscles has prompted several studies in this laboratory, the results of which support our hypothesis that the proprioceptors of the intercostal musculature serve to integrate its two main functions respiration as well as posture and movements of the trunk The diaphragm on the other hand would seem to lack such a marked dual function (Corda Eklund and Euler 1965 Corda *et al* 1965*b*, Euler 1966*a* *b*

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Another approach towards a full understanding of this difference in proprioceptive innervation of the intercostal muscles and the diaphragm would be to investigate whether intercostal muscle receptors are, to any significant extent, engaged in the control of phrenic activity In previous work it has been shown that subjecting the external intercostal musculature and the diaphragm to the same increment in load, by occluding the airways provokes a compensatory autogenetic increase in excitation of the intercostal motoneurones (the load compensating reflex' of Corda *et al* 1965a, *cf* also Euler 1966*a*, *b*) This effect, however, was not transmitted to the phrenic motoneurone pool as a whole This fact, confirmed for the rabbit by Sant'-Ambrogio and Widdicombe (1965), does not, however, rule out the possibility that some local intercostal to-phrenic reflexes are of importance for the interaction between the diaphragm and those trunk wall muscles which are closely related to attachment of the diaphragm

In fact a few reports in the literature indicate that phrenic motoneurones can be reflexly influenced by mechanical stimuli applied to the trunk wall (Garcia Ramos and Lopez Mendoza 1959) and that stimulation of a whole intercostal nerve may cause a reflex discharge also in the phrenic nerve (Downman 1955) The present and two subsequent papers deal with a study of intercostal to-phrenic reflex effects on spinal and decerebrate preparations (Decima and Euler 1968*a* *b*) A short report of some of the results has been published elsewhere (Decima Euler and Thoden 1967)

Methods

The experiments were performed on spinal cats and rabbits Initial surgical procedures were carried out under ether anesthesia After cannulation of the trachea the C5 phrenic root of one side was dissected and severed as far distally as possible The spinal cord was cut at the level of C1 and the animal was put under artificial respiration The deep back muscles were removed between T7 and L1 and according with the aim of the particular experiment see Results) different branches of the thoracic nerves (*e g* external intercostal internal intercostal dorsal rami etc) were dissected close to the vertebral column cut distally and mounted for recording or stimulation

In the experiments designed to study the afferent fibre groups responsible for the phrenic reflex a thoracic laminectomy was performed The cord was protected by warm mineral oil The afferent volley was recorded at the entry of the intact dorsal root by means of a fine silver electrode carefully introduced between the cord dorsum and the dorsal rootlets In some experiments a rootlet was cut centrally and placed on hook electrodes in order to achieve a larger signal to noise ratio

The electromyographic activity of the diaphragm was recorded by inserting fine indwelling electrodes (enammeled with 1 mm at the tip left bare) with the technique described by Basmajian (1962) Phrenic motor discharge was monitored either in the whole but desheathed C5 phrenic root or in dissected filaments of the same root with only a few active motor fibres An RC-network was used to integrate the phrenic and intercostal motor discharges and the magnitude of the response was assessed by measuring the area of these integrated records Tracheal pressure and abdominal pressure were recorded with strain gauge manometers Tidal volume was monitored by means of a bag and box system and a small Krogh

are given with the Results

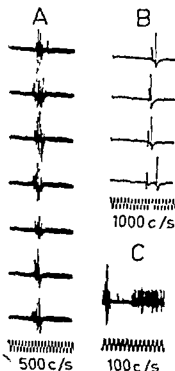


Fig. 1. Records obtained from the central end of the left C5 phrenic root in response to stimuli applied to the ipsilateral 10th external intercostal nerve. *A*. A series of consecutive sweep records from the whole root. Single shocks of supramaximal strength at a rate of 1.2 per sec. Note the spontaneous fluctuations in response magnitude. *B*. Records from a thin filament of the central end of the C5 root containing two active fibres. Faster sweeps otherwise as in A. *C*. From a coarse filament of the central end of phrenic C5 root exhibiting some 'spontaneous' tonic efferent activity. 15 superimposed sweep records at low sweep speed showing the initial reflex volley followed by a period of depression of the tonic activity. Stimulus conditions as in A.

Results

The basic intercostal-to-phrenic reflex dealt with in this paper is shown in *A* of Fig. 1. The records of this Figure were obtained from the central end of the whole left C5 phrenic root in response to a single shock of maximal strength to the 10th external intercostal nerve. It resembles in several aspects the intercostal-to-intercostal reflex described in detail by Downman and his collaborators (Downman 1955, Downman and Hussain 1958, Alderson and Downman 1966).

The intercostal-to-phrenic responses which could be elicited both from the external and from the internal intercostal nerve (see below) showed marked 'spontaneous' fluctuations in size as seen in the consecutive sweeps of Fig. 1*A*. These fluctuations showed no correlation with the strokes of the respirator. The latency of the response was usually about 12 msec. In single unit records of which Fig. 1*B* is an example, latencies as short as 10 msec have been observed (*cf.* Downman 1955). The lantency was generally inversely related to the response magnitude and was thus dependent on 1) the stimulus strength, 2) variations in excitability of the reflex arc and 3) the nerve stimulated (see below).

The duration of the response indicates an appreciable temporal dispersion occurring somewhere in the arc including the motoneurone itself. Results obtained from experiments on single phrenic efferents indicate, however, that repetitive firing of individual motoneurones occurred only rarely and thus was of no significance for the duration of the volley (Fig. 1*B*).

In the spinal preparation some 'spontaneous' activity was usually recorded from the efferent phrenic fibres indicating that the phrenic motoneurones are in fact receiving a tonic excitatory input from sources at spinal levels Correspondingly, electromyographic recording from the diaphragm of such preparations often showed low level tonic activity, provided that the phrenic nerves were intact Against such a background of tonic activity, the reflex elicited from one of the lower intercostal nerves showed that the volley was often followed by a period of depression lasting for some 35—55 msec (*cf* Fig 1*C*) Since this depression could be expected to be due to the recovery cycle of phrenic motoneurones (Gill and Kuno 1963, Decima and Euler 1968*a*), it is not clear yet if an active inhibition is present as well A true inhibition however, was often a prominent feature of the response in the decerebrate preparation (Decima and Euler 1968*b*, *cf* also Alderson and Downman 1966)

In response to intercostal stimulation, the phrenic reflex was obtained equally well and with the same latency from both the ipsilateral and the contralateral phrenic nerve By successive lesioning of the spinal cord in acute experiments, the ascending pathways from the lower thoracic segments to the phrenic motor nucleus in C5—C6 (C7) were found to run bilaterally in the ventrolateral funiculi.

The phrenic response to intercostal stimulation was found to be quite sensitive to pentobarbital A dose as small as 13 mg/kg for instance, reduced the response to less than 20 % of control values

Receptive field

The biggest responses have been obtained from the lower intercostal nerves and from the dorsal rami of the thoracic spinal nerves Weaker responses were obtained from the lumbar nerves supplying the abdominal muscles and from the great splanchnic

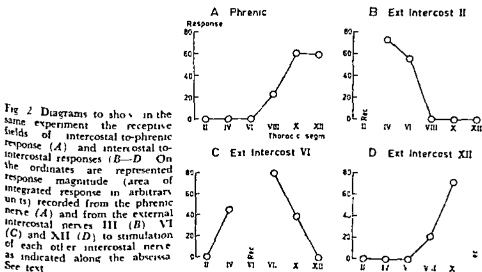


Fig 2 Diagrams to show in the same experiment the receptive fields of intercostal to-phrenic response (*A*) and intercostal to-intercostal responses (*B*—*D* On the ordinates are represented response magnitude (area of integrated response in arbitrary units) recorded from the phrenic nerve (*A*) and from the external intercostal nerves III (*B*) VI (*C*) and XII (*D*) to stimulation of each other intercostal nerve as indicated along the abscissa See text

nerve Fig 2*A* gives examples of results obtained from experiments on the responsiveness of the phrenic motoneurones to ipsilateral stimulation of the external intercostal nerves from above down In these studies the intercostal to-phrenic responses were compared in the same experiment with intercostal to-intercostal responses evoked by stimulation of the various external intercostal nerves and recorded in every second of the other external intercostal nerves In Fig 2 records from only intercostal II, VI and XII have been selected for comparison with the phrenic response As can be seen in *A* the largest intercostal to-phrenic responses were evoked from the nerves of the lowest external intercostal muscles *i e* those influencing the ribs at which the costal parts of the diaphragm are attached The upper external intercostal nerves although anatomically much closer to the phrenic nucleus (C3—C6) failed to evoke any significant reflex responses in the phrenic nerve In contrast the intercostal to-intercostal reflexes gave the largest response and the shortest latencies between neighbouring intercostal nerves independent of the segmental level (*cf* Fig 2*B* *C* and *D*) When stimulating segments farther away from the recording site the response decreased in magnitude whereas the latency increased in general agreement with the results of Downman (1955) and of Sumi (1963) The results thus show that the receptive area for the external intercostal to-phrenic reflex coincides with the region of the anatomical insertion of the diaphragm

Both the external and the internal intercostal nerves yielded strong phrenic responses It should be recalled that the external intercostal nerve is a pure muscle nerve innervating the external intercostal muscle The internal intercostal nerve on the other hand gives off the lateral and anterior perforating cutaneous nerves as well as muscle branches both for the internal intercostal muscle and for abdominal and certain other muscles of the trunk wall No phrenic reflex was ever obtained in response to the cutaneous branches although they are considerably thicker than the muscle branches from which prominent reflexes were evoked

Thresholds

The thresholds of the intercostal to-phrenic reflex have been studied in relation both to the threshold for intercostal and abdominal muscle reflexes and to the threshold for the different afferent fibre groups as estimated from the composite potential of the arriving volley at the dorsal root entry

The intercostal to-phrenic response proved to have the same threshold as the *intercostal to-intercostal reflex recorded from a neighbouring segment* At roughly the same threshold a reflex contraction appeared also in some abdominal muscles The threshold and the stimulus to response relationship for the intercostal to-phrenic reflex remained unaltered after paralyzing doses of tubocurarine or gallamine (Flaxedil® May and Baker) proving that the phrenic response described so far did not depend on secondary engagement of peripheral loops involving muscular contractions and muscle receptors In order to exclude the possibility of excitation being transmitted *via* recurrent collaterals from the lower intercostal motoneurones to the phrenic motor nucleus the elicitation of the intercostal to-phrenic reflex was

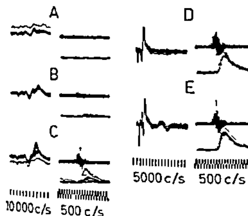


Fig 3 To compare the mass action potentials at the dorsal root entry (left records in each pair) with the phrenic responses (to the right) recorded directly (upper traces) and integrated (lower traces) Single shocks to internal intercostal X at 0 3 shocks per sec Ten superimposed sweeps *A* Threshold for dorsal root response, *B* 3db, *C* 4 db, *D* 20 db and *E* 26 db above threshold (*A*)

tested after severing the corresponding ventral root This had no effect on the phrenic response After cutting the dorsal root, however, the response was completely abolished The threshold for phrenic responses to external intercostal stimulation was always 6 to 10 db higher than stimulation of the internal intercostal nerve

The thresholds of the intercostal-to-phrenic responses have also been determined in relation to the incoming dorsal root volley The reflex and the mass action potentials at the dorsal root entry were simultaneously recorded In the internal intercostal nerves the fastest conducting fibres (group Ia) had conduction velocities ranging from 115 to 90 m/sec At strengths 2 to 4 db above threshold for the fastest fibres the phrenic reflex discharge appeared simultaneously with a second hump in the incoming dorsal root volley The conduction velocities corresponding to this second hump, the tail end of which grew together with the reflex response with increasing intensity up to about 6 to 12db above threshold was between 80 and 50 m/sec These results from the internal intercostal nerve suggest that the phrenic response appeared and grew when the fibre groups Ib and II became engaged

In Fig 3, from the 10th internal intercostal nerve the dorsal root responses are shown to the left in each pair To the right are the phrenic responses recorded directly (uper tracings) and as integrated activity (lower tracings) At threshold strength for the earliest component no reflex response was visible (Fig 3*A*) An increase of 3 db in stimulus strength however sufficed to elicit a phrenic discharge and a second hump in the dorsal root response (*B*) The two dorsal root components fused together as they grew with increasing stimulus strength (*C*) the phrenic response grew correspondingly The conduction velocity for the fibres of the early component was about 100 m/sec and for those of the second hump was 70—50 m/sec

At considerably higher stimulus strength (20 to 26 db above threshold for dorsal root response) a late wave appeared, the conduction velocity of this fibre group was about 20 m/sec However the recruitment of this fibre group did not produce a detectable increase of the phrenic response (*cf* Fig 3*D* and *E*)

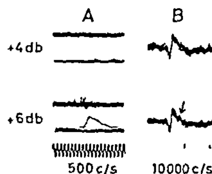


Fig 4 To compare the phrenic responses (*A*) and the mass action potentials at the dorsal root entry (*B*). Each record is compared at ten superimposed frames at a frequency of 0.3 per sec. In *A* the upper traces in each pair are the direct records and the lower traces are the integrated responses. Single shocks to external intercostal XI. Stimulus strength in db above threshold for dorsal root response. See text.

The results for stimulation of the external intercostal nerve have been slightly different in that the phrenic reflex did not appear until the stimulus strength had been increased 6 to 10 db above threshold for the dorsal root volley. This indicates that no phrenic reflex was elicited from the external intercostal nerve until group II afferents became stimulated. The records of Fig. 4 are from an experiment on this nerve. The phrenic responses in *A* and the arriving dorsal root volleys in *B* at faster sweep speeds were recorded simultaneously. At 6 db above threshold for the fastest conducting fibres the threshold for the reflex was just reached (lower records of column *A*). At this intensity a second wave in the dorsal root potential appeared (marked by the arrow in the lower record of *B*). The latencies of the two waves in the dorsal root neurogram correspond to conduction velocities of 120 and 65 m/sec respectively.

Mechanical stimuli

Various types of mechanical stimuli applied to the lower thoracic region such as tapping or pressing on the back muscles or on the lower part of the rib cage proved very effective in provoking an increase in phrenic activity. This is in general agreement with some observations reported by Garcia Ramos and Lopez Mendoza (1959) although these authors did not specify the regions from where the effects on the diaphragm could be elicited. In the present work it was found that tapping or pressing on the upper part of the rib cage did not generally elicit phrenic reflex effects. This is consistent with the negative results of electrical stimulation of upper intercostal nerves mentioned above.

Intercostal-to-phrenic reflex activation was found not only to be a phasic affair but tonic as well. Sustained pressure on the lower part of the rib cage elicited a phrenic discharge which usually lasted as long as the stimulus was maintained; however the initial part of the response usually contained a phasic burst which gradually declined to the level of the sustained tonic part of the response. Fig. 5 gives some examples of such mechanically elicited phrenic reflexes in the spinal cat (*A* and *C*) and rabbit (*B*).

In this connection mention should be made of the fact that in the spinal prepara-

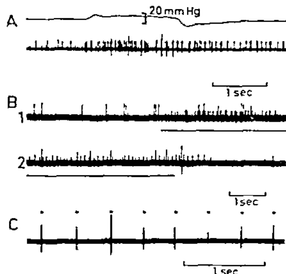


Fig 5 Examples of efferent phrenic responses to different mechanical stimuli applied to the trunk wall *A* Spinal cat. Thin filament of C5 phrenic root Pressing on the upper part of the abdominal wall as indicated by the change in intratracheal pressure (Respiratory pump momentarily stopped and the tracheal cannula closed) *B* Spinal rabbit Thin filament of C5 phrenic root Maintained light pressure over the lower part of the thorax as marked Records of 1 3 sec omitted between record 1 and 2 *C* Spinal cat Coarse filament of C5 phrenic root Tapping on the lower dorsal part of the thorax as marked

tion the 'spontaneous' phrenic activity was never seen to be modulated in phase with artificial ventilation even at big stroke volumes Nor did the spontaneous' variations in the responsivenes of the phrenic motoneurones, mentioned earlier in this paper, show any relations to the cycle of the respiratory pump

Succinylcholine

In order to obtain further information concerning the type of muscle receptors responsible for the elicitation of the phrenic reflex, the effects of succinylcholine were tested This drug is known to stimulate selectively the muscle spindles (Granit, Skoglund and Thesleff 1953 see Fehr 1965 also for further references) driving their primary endings at higher firing rates than the secondary endings (Fehr 1965 Rack and Westbury 1966, Smith 1966) The Golgi tendon organs are silenced as a conse quence of the paralysis

Close arterial injection of succinylcholine (0 25 mg of Celocurine iodide® Vitrum') into the descending aorta was performed by means of a Teflon catheter inserted through the femoral artery The catheter was advanced until its tip reached the level of T8 The aorta was clamped at the level of T13 during the period of injection In these experiments the internal intercostal nerves and the dorsal rami of the segments T8—T13 had been severed leaving only the external intercostal nerves intact Injections of succinylcholine in such a preparation elicited an efferent phrenic discharge of short duration which thus can be attributed to an increased spindle discharge in the external intercostal muscles of the segments T8—T12 A further reduction of the possible receptive area by complete denervation of the segments of one side (leaving only the external intercostal nerves of T8—T13 on the other side intact) left a weak though significant response to succinylcholine

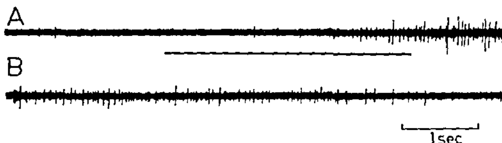


Fig 6 Efferent phrenic response from the field innervated by external intercostal nerves T8—T12 to close arterial injection of succinylcholine Duration of injection marked See text

Fig 6) The short duration of the response to succinylcholine from the external intercostal muscles suggests that mainly secondary spindle endings were responsible for this phrenic response A considerably stronger phrenic response was obtained when instead the internal nerves were left intact (i e external nerves and dorsal rami branches were cut) Since the internal intercostal nerve innervates a larger muscle mass than the external nerve it seems likely that it carries also a larger number of secondary spindle afferents Thus the larger response from the internal nerves to succinylcholine does not indicate that primary spindle endings innervated by these nerves contributed to the reflex effects

Vibration

It has been known for some time that the ability to follow high frequency vibration is a property which distinguishes the primary muscle spindle from the secondary endings and Golgi tendon organs (Bianconi and Van Der Meulen 1963 Euler and Peretti 1966 Brown Engberg and Matthews 1967) These specific effects of vibration were used in a series of experiments to elucidate further the type of muscle receptors involved in the production of the intercostal to-phrenic reflex Vibration was applied after selective denervation of one or two of the different muscle groups investigated (external intercostal internal intercostal and deep paravertebral muscles) These experiments showed clearly that excitatory reflex effects on the phrenic motoneurones could be obtained from vibration applied to the dorsal part of the trunk provided that the innervation of the paravertebral back muscles was at least partly left intact When afferent input from these muscles could be excluded vibrations applied to different parts of the rib cage did not give rise to any significant phrenic effects

These results indicate that primary muscle spindle endings in neither the external nor the internal intercostal muscle participate to any significant extent in the intercostal to-phrenic reflex It is of considerable interest however that similar reflex effects from the backmuscles may be elicited from their primary spindle endings However, this has not been analyzed any further

Discussion

The intercostal to-phrenic reflex proved to have several features in common with the intercostal to-intercostal reflex described by Downman (1965). However, the latency for the latter reflex as given by Downman is considerably shorter than that obtained here for the intercostal to-phrenic reflex. Downman's figures are also smaller than those obtained in the present work for the intercostal-to-intercostal reflex (Downman used considerably higher stimulus strengths.) Another difference between the conclusions reached by Downman concerning the intercostal to-intercostal reflex and our results on the intercostal to-phrenic reflex is that we could not obtain any evidence for repetitive firing of any one phrenic motoneurone in the early response volley.

The most important difference between the phrenic and the intercostal responses to intercostal stimuli concerns their receptive areas: the phrenic motor nucleus in the 5th and 6th cervical segments showed a responsiveness to intercostal stimulation which resembles that of the intercostal nerves of the 10th to 12th thoracic segments. In contrast to the inter intercostal reflexes the phrenic motoneurone pool did not show signs of reflex connections from the neighbouring segments. On the contrary, it was found that the diaphragm had the most potent reflex connections with those muscles with which, in the course of its evolution, it had established functional and anatomical interplay (*cf* Wells 1954). The anatomical difference in the organization of the receptive areas of the inter intercostal and the intercostal to-phrenic reflexes can thus be regarded as a similarity from a functional point of view: the phrenic motoneurone pool showed a responsiveness to intercostal stimuli which resembled that of the lowest intercostal motor nuclei.

Similar reflex effects were obtained in response to stimulation of the internal and external intercostal nerves. In the internal nerve, however, both tendon organs and secondary muscle spindle endings appeared to contribute to the reflex, whereas in the external nerves it seems to be merely the secondary muscle spindle endings which gave rise to the phrenic reflex.

This conclusion is based on four lines of evidence: 1) The considerably higher threshold for external than for internal intercostal reflex responses. 2) The engagement of group Ib fibres at threshold and group II fibres at further growth of internal intercostal reflex response, whereas in the external intercostal nerve group II fibres had to be engaged before any reflex response appeared. 3) Administration of succinylcholine caused phrenic reflex response after selective denervation of either the external or the internal intercostal nerve. 4) After similar selective denervations vibration did not elicit phrenic reflex responses either from the external or from the internal intercostal muscles.

The functional significance of the difference in afferent input from the two sets of muscles is not clear. It is suggestive, however, that expiratory actions of internal intercostal and abdominal muscles, particular in forceful expulsive actions, may need a strong protective reflex contraction of the diaphragm (Agostoni and Torri 19

cf also Jodat, Horgan and Lange 1966) The participation of the internal intercostal tendon organs in producing reflex contraction of the diaphragm fits the general scheme established for those receptors in as far as it concerns their excitatory effect on antagonistic muscles

The somewhat surprising result that group II afferents from both the synergistic external intercostal and the antagonistic internal intercostal abdominal muscles exert similar excitatory effects on the diaphragm should probably be looked upon against the background that the two sets of intercostal muscles can work in synergism in postural reflexes (*cf* Corda *et al* 1966) and probably also under any such circumstances which involves a need for increased stiffnes of the thoracic cage or part of it.

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Excitability of Phrenic Motoneurones to Afferent Input from Lower Intercostal Nerves in the Spinal Cat

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Abstract

DECIMA E E and C v EULER *Excitability of phrenic motoneurones to afferent input from lower intercostal nerves in the spinal cat* Acta physiol scand 1969 75 580—591

The reflex connections between the lower intercostal segments and the phrenic motor pool described in a preceding paper, has been studied further It was found that the diaphragm on contraction could exert enough mechanical force on the lower part of the rib cage to evoke a reflex discharge in the phrenic motoneurones The intercostal to-phrenic reflex arch thus constitutes a link in a loop for self-excitation of the phrenic nucleus which may make up for its lack of autogenetic facilitation Up to five synchronized reverberating phrenic volleys could be recorded in response to a single diaphragmatic jerk These were abolished by paralyzing doses of gallamine Conduction times and excitability cycles for the loop have been studied Examination of reflex firing probality for phrenic motoneurones has revealed considerable individual differencies in their responsiveness to each of the three afferent input parameters a) nerve stimulated b) strength and c) frequency of stimulation The order of reflex recruitment of phrenic motoneurones followed the same order as found for their respiratory engagement small spikes always appeared earlier than bigger spikes

In a preceding paper (Decima Euler and Thoden 1968) it was shown that the diaphragm which in itself has a very scanty supply of muscle spindles and which seems to lack autogenetic facilitation (Corda Euler and Lennerstrand 1965 also for further references) is under some excitatory control from proprioceptors in the lower intercostal muscles i e in the part of the trunk wall on which the diaphragm inserts It was shown that various types of mechanical stimuli such as pressing or tapping on the lower ribs evoked discharges in the phrenic nerve In the present paper it will be shown that contractions of the diaphragm itself can exert such mechanical forces on the trunk wall which in turn may provoke a reflex discharge in the phrenic nerve, thus providing evidence for a mechanism of self facilitation Some details of the operation of the circuits involved have been analyzed such as loop times excitability properties of the intercostal to-phrenic reflex arc and reverberating periods A brief report of some of these results has been published elsewhere (Decima Euler and Thoden 1967

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Methods

The surgical procedures and techniques used will be described briefly since they have been reported in more detail previously (Decima *et al* 1968) All experiments were done in adult cats with anemic destruction of the brain and the spinal cord severed at the level of C1 Branches of different thoracic and lumbar nerves were prepared for stimulation and recording Phrenic motoneuron activity was monitored by recording either the diaphragmatic electromyogram or the centrifugal discharges in the C5 phrenic root in the neck In some experiments stimulating electrodes were placed on one or both phrenic nerves in the neck distal to the junction of its C5 and C6 roots

A thoracic laminectomy was performed in the experiments designed to study the different type of intercostal muscle receptor being activated by diaphragmatic contraction Spindles and Golgi tendon organs were identified by their different responses to muscle contractions For this purpose stimulating electrodes of the type used by Critchlow and Euler (1963) were placed on the ventral roots of the segments under study

The magnitude of the phrenic reflex responses (when recorded in the whole C5 phrenic root) was assessed by measuring the area of the integrated responses

Some special details of the technique will be given with the Results

Results

Phrenic self facilitation by the diaphragm-intercostal loop

Figure 1 gives different examples of efferent phrenic responses to diaphragmatic contraction elicited by tetanic stimuli applied to the peripheral ends of both severed phrenic nerves (See the schematic drawing of Fig 3) Records *A* and *C* of Fig 1 were obtained from a filament containing only a few fibres The contraction of the diaphragm signalled by the negative pressure deflection caused a marked increase in the phrenic discharge which outlasted the tetanic stimulus The stronger effect in *A* was produced by a stimulus strength 2 db higher than that employed in *C*

The phrenic response was produced by an increased activity of the motoneurones engaged in the background activity commonly observed in the spinal preparations, and by recruiting new motoneurones Characteristically, the freshly recruited spikes were of bigger amplitude, showing that by reflex activation produced in this way the large motoneurones have higher threshold and less tendency to fire tonically than the smaller ones This pattern of recruitment according to size has recently been

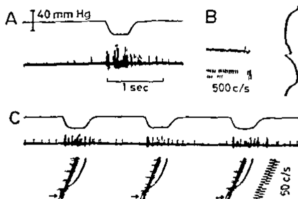


Fig 1 Efferent phrenic-to-phrenic responses produced by tetanic stimulation of the peripheral stumps of both cut phrenic nerves Respiratory pump momentarily stopped and tracheal cannula closed The diaphragmatic contractions are indicated by the negative deflections of the intratracheal pressure (upper tracings in *A* and *C* and to the right in *B*) Records from thin filament of the central end of the phrenic C5 root Stimulus strength 2 db higher in *A* than in *C* Arrows in the sweep records of *C* indicate beginning of tetani For further explanation see text

found also for the respiratory drive of the phrenic motoneurones (Yaşargil 1967) and appears to be the rule in the lumbo sacral segments (*e g* Denny Brown 1929 Granit, Henatsch and Steg 1956, Henneman Somjen and Carpenter 1965)

Loop times

The latencies from the beginning of the centrifugal phrenic stimulus have ranged from 21—25 msec This is shown in *B* and the sweep records of *C* in Fig 1 The latter records also show the tendency of phrenic motoneurones to discharge rhythmically (*cf* Adrian and Bronk 1928) with a period of some 60 msec

Other temporal features involved in this loop have been studied in experiments with electromyographic recording from both hemidiaphragms and stimulation of the intact right phrenic nerve in the neck The direct volley thus elicited in the right hemidiaphragm often exhibited some temporal dispersion This is probably due both to the differences in motor fibre size of the phrenic nerve (Hinsey, Hare and Phillips 1939, Landau Akert and Roberts 1962) and to the effect of the fanning out of the motor fibres (*cf* Eccles and O Connor 1939) over the surface of the diaphragm (Strauss 1933) The first reflex response was obtained from the contralateral hemidiaphragm after a latency of 25—30 msec, which, of course is slightly longer than that measured for the efferent phrenic volley recorded in the neck In both hemidiaphragms however a late reflex volley generally appeared after a total latency period of 55—70 msec After the stimulated phrenic nerve had been cut proximally the site of the stimulating electrodes both the early and the late reflex responses on the contralateral side were still recorded with the same latencies as before whereas the reflex discharge on the ipsilateral side was completely abolished

In order to get further information about the time table for the phrenic reflex elicited indirectly by diaphragmatic contractions the corresponding afferent input to the cord was monitored in the dorsal roots of the lower thoracic segments together with the direct electromyographic response from the diaphragm In some of these experiments the afferent mass discharge in a whole rootlet was recorded so as to obtain an average of the afferent input from the various receptors in a relatively big area of an intercostal space In other experiments the responses from individual receptors were recorded from split dorsal root filaments In these experiments either the internal or the external intercostal nerves had been severed to ascertain that the arriving message was coming only from either the external intercostal or the internal intercostal abdominal muscle receptors respectively

The earliest afferent impulses were recorded in the dorsal root 5—6 msec after the shock to the phrenic nerve In superimposed records from rootlets such as that of Fig 2 *A*, the latency was usually found to be 6—8 msec for the external intercostal afferents and 7—9 msec for the afferents of the internal intercostal nerve This early afferent volley generally had a duration of 6—11 msec Figure 2 *B* is an example of records of receptor afferents contributing to the early volley Identification of the receptors was based on their different responses to succinylcholine (Fehr 1965 Smith 1966) Such records obtained from fine dorsal root filaments

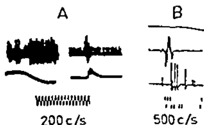


Fig 2 Intercostal afferent responses in dorsal root filaments to diaphragmatic jerks Single shocks to the distal stumps of both phrenic nerves *A* 30 superimposed sweep records To the left upper tracing is from a fine dorsal root filament at T11 showing the responses from external intercostal receptors (the internal intercostal nerve of this segment being cut) The contraction of the diaphragm is signaled by the intratracheal pressure deflection To the right in *A* is shown the ipsilateral phrenic response directly recorded (upper) and integrated response (lower tracing) simultaneously recorded from the proximal stumps of the phrenic C5 root *B* Uppermost tracing intratracheal pressure change Middle tracing the direct electromyographic response from the diaphragm Lower tracing afferent responses from internal intercostal primary (large spike) and secondary muscle spindle ending (smallest spike) Intermediate spike is from a Golgi tendon organ

showed that the early afferent volley usually did not consist of more than two or three spikes in any single afferent fibre A temporal dispersion of 1—2 msec at the dorsal root must be taken into account allowing for the difference in centripetal conduction time for receptors located in different parts of the intercostal spaces

The movements of the ribs caused by the pull of the diphragm are fairly complex Records from individual afferents have demonstrated however, that contributions to the early volley were coming from primary and secondary spindle afferents of both the internal and the external intercostal nerves as well as from tendon organ afferents of the internal intercostal nerve

In the preceding paper it was found that the shortest latency for the phrenic response to stimulation of the intercostal nerve at a site close to the vertebral column, was about 12 msec (10—14 msec) If this figure is subtracted from the latency of about 22 msec for ⁻ whole phrenic to-phrenic reflex it will give a latency

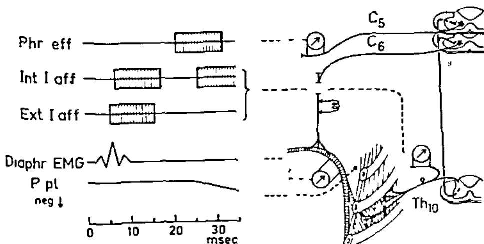


Fig 3 To show the time relations between the direct electromyographic response of the diaphragm the afferent volleys in the dorsal root and the phrenic reflex response To the right is shown schematically the corresponding experimental situation

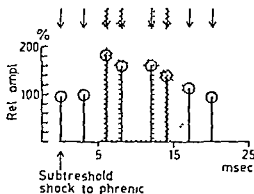


Fig 4 To show the increase in responsiveness of the phrenic pool to test shocks to the 10th intercostal nerve during the period of the early afferent volley (stippled) following a subthreshold twitch of the diaphragm provoked by a single shock to the peripheral ends of the cut phrenic nerves

of about 10 msec for the arrival to the dorsal roots of the volley producing phrenic excitation some 12 msec later

These time relations summarized in a diagrammatic form in Fig 3, definitely tie the phrenic response to the early discharge either in the external or in the internal intercostal afferents or in both. The hypothesis that the early afferent volley is exciting the phrenic motoneurone pool was brought to a test in experiments of the type illustrated in Fig 4. A twitch of the diaphragm of subthreshold strength for elicitation of a manifested centrifugal phrenic discharge acted as the conditioning input against which the responses of centripetal single shock stimuli to the 10th intercostal nerve were tested. The responses of the test shocks delivered during the period of the conditioning early afferent discharge (produced by diaphragmatic contraction) were clearly facilitated with the strongest effect in the first part of the early afferent volley

Following the early discharge the internal intercostal afferents generally showed a short pause or at least a marked decrease in firing rate lasting on an average for about 8 msec after which the afferent discharge reappeared or increased again. The leading edge of this second discharge might constitute the afferent input responsible for a less constant second phrenic volley occurring if at all some 14 msec later. Sometimes a third and even a fourth volley appeared

The records of external intercostal afferents were less clear. A long pause of about 35 msec was often observed after the early discharge

Ventral root stimulation

Another, indirect way to evoke an intercostal-to-phrenic reflex discharge was to elicit contractions in the intercostal muscles by stimulation of one or more of the lower thoracic ventral roots

In these experiments either the internal or the external intercostal nerves were severed. Both an early and a late phrenic reflex discharge was obtained in response to ventral root stimulation. The latency period of the early response was 21–25 msec whereas that of the late discharge ranged between 40 and 46 msec The

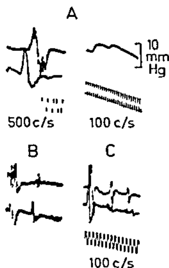


Fig 5 Electromyogram (EMG) of the reflex responses of the diaphragm (upper records in each pair) and the abdominal rectus muscle (lower records) to afferent stimulation of internal intercostal XI *A* To the left are the EMG responses at fast sweep speed and to the right the simultaneously recorded intra-abdominal pressure at slow sweep speed showing double pressure peaks *B* Same as *A* (except pressure) at slow sweep speed to show second discharge *C* Same as *B* but at higher stimulus strength (+4db) showing also a third discharge See text for further explanation

latencies for the early reflex discharge were almost the same irrespective of which group of muscles was activated and almost the same as for diaphragmatic contraction

Recurring phrenic volleys

When stimulating an intercostal nerve the response was evoked not only in the phrenic nerve but also in some other muscles of the trunk wall In Fig 5 the response in the electromyogram of the diaphragm is recorded together with the electrical activity of the abdominal rectus muscle *A* of this Figure shows the difference in the latencies between these two responses (6 msec) probably accounted for by the longer conduction distance involved in the phrenic response *B* of the same Figure at slower sweep speed shows that a second reverberating wave appeared in both muscles The interval between the first and the second wave was found to be dependent on the stimulus strength i e the magnitude of the first response

Latencies below 50 msec were only occasionally recorded for the responses of these muscles In Fig 5 *B* this minimal value was almost reached for the abdominal rectus muscle but not for the diaphragm (58 and 66 msec re pec ively At higher stimulus strength (Fig 5 *C*) the intervals for both muscles became almost equal 52 msec for the rectus muscle and 53 msec for the diaphragm Now even a third wave appeared in both muscles again almost synchron usly It should be observed that this loop-time of about 50 msec is of the sam orde as those obtained for the second and following phrenic volleys triggered by stimulating the distal ends of the phrenic nerves as described above These recurring phrenic respon es were abolished by paralyzing doses of tubocurarine or gallamine (Flaxedl® May & Barker) This indicates that they are not intrinsic spinal phenomena but depend on muscle lin

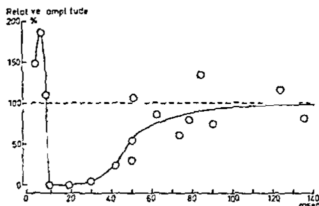


Fig 6 Recovery cycle of the intercostal to phrenic reflex studied by paired shocks to internal intercostal nerve X. Each point represents the average area of an integrated phrenic reflex response expressed in per cent of control values

Excitability properties of the intercostal to-phrenic reflex arc

The self facilitatory and reverberating activity shown by the experiments described above prompted a study of the excitability properties of the intercostal to-phrenic reflex arc

The recovery cycle of the reflex was analyzed by the technique of paired stimuli to an intercostal nerve Fig 6, from such an experiment, shows a depression of the excitability lasting for about 50 msec A similar time course was obtained with intracellular technique by Gill and Kuno (1963) for the recovery following antidromic activation of phrenic motoneurones It is thus suggestive that the recovery cycle obtained in the present experiments is mainly dependent on the phrenic motoneurones (*cf* Pitts 1943) and may explain the post excitatory depression described in a preceding paper (Decima *et al* 1968) The refractory period, shown in the diagram to last at least 40 msec would clearly be a limiting factor for the maximum frequency of a reverberating activity In fact, the shortest period obtained in this type of activity was around 50 msec as shown above

On top of the spontaneous fluctuations in excitability mentioned in the preceding paper (Decima *et al* 1968) repetitive stimulation of one of the lower intercostal nerves at different rates caused accumulated changes in the responsiveness of the phrenic motoneurones These could not be predicted simply from the recovery cycle described above obtained by paired shocks Therefore the excitability of single phrenic motoneurones activated by the intercostal to-phrenic reflex has been studied with respect to possible changes in their firing probability when different input parameters were varied The three parameters were 1) stimulus strength 2) stimulus frequency and 3) segmental level i e the intercostal or lumbar nerve being stimulated The excitability generally decreased with increasing rate of stimulation. This depressive effect of increased stimulus frequency could within limits be matched by an increased strength of stimulation The firing probability of one motoneurone, for instance was reduced from 100 % to 11 % merely by raising the repetition rate from 0 6 per sec to 14 5 per sec It was again restored to the 100 % level by increasing the stimulus strength by not more than 4 db

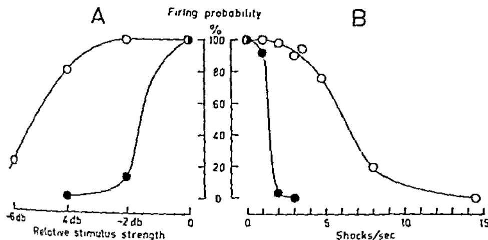


Fig 7 Differences on reflex firing probability of different phrenic motoneurones caused by changes in *A* stimulus strength and *B* in stimulus frequency Open circles represent small amplitude spike and filled circles large amplitude spike, recorded in left C5 phrenic root. See text

When two spikes of different amplitude (*i e* different axonal diameters) were recorded in a single filament the smallest spike always showed the lowest threshold to intercostal stimulation This is shown diagrammatically in Fig 7 *A* where the firing probability is related to stimulus strength Also for a given intensity of stimulation the firing probability of large spikes started to drop at a lower stimulus frequency than did the smaller spikes This is exemplified in Fig 7 *B* In accordance with these results it was also found that the small spikes generally had shorter latencies than the larger ones Examples of some kind of specific input-output relation have also been observed with regard to the change in responsiveness produced by altering the rate of stimulation of different nerves Fig 8 is collected from an experiment in which stimulation of a lumbar nerve branch (for the abdominal wall) at maximal strength and at a rate of 7 shocks/sec (upper part of *A*) proved to be somewhat less effective in driving the phrenic motoneurones sampled in the filament, than was stimulation of an intercostal nerve (upper part of *B*) This relation was reversed however, when the rate of stimulation was increased to 34 per sec (*cf* lower parts of *A* and B of Fig 8)

Such differential effects on members of the phrenic motor pool produced by stimulation of various nerves are also illustrated in Fig 9 *A* which gives the firing probability of a unit as a function of stimulus frequency when different nerves were stimulated The responses of this unit to stimulation of the XIth intercostal and the Ist lumbar nerve (lower branch) are qualitatively similar the only difference being the critical frequency at which the firing probability starts to drop below 100 % However stimulation of the upper branch of the Ist lumbar nerve produced a different effect not only did the unit show a firing probability below 100 % at the

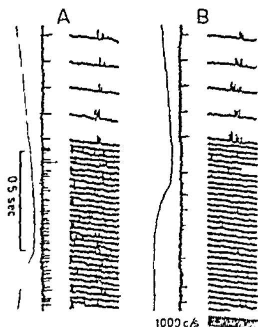


Fig 8

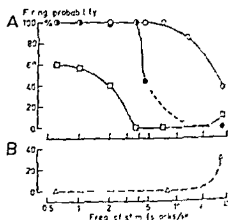


Fig 9

Fig 8 Continuous and sweep records from left phrenic C5 and intratracheal pressure to show changes in responsiveness of phrenic motoneurones to changes in the rate of stimulation applied to two different nerves *A* Phrenic responses to stimulation of abdominal muscle branch of 1st lumbar nerve first at 7 shocks per sec and then at 34 per sec *B* Phrenic responses to stimulation of the 11th internal intercostal nerve at 7 per sec and 34 per sec See text

Fig 9 *A* Differences in the relation between firing probability of a phrenic motoneurone and stimulus frequency when stimulating different nerves Open circles 11th external intercostal nerve Filled circles lower branch of 1st lumbar nerve Squares upper branch of 1st lumbar nerve *B* From another phrenic motoneurone which was driven by stimulation of the lower branch of the 1st lumbar nerve at 34 shocks per sec but was not driven at 0.6 and 8 shocks per sec See text

lower stimulus rates but the responsiveness increased again markedly after passing through a range of critical stimulus rates at which the probability of firing was zero Fig 9 *B* from another filament shows an example of what could be described as an extreme case of the same phenomenon Although the unit did not respond at all to stimulus frequencies of 0.6 per sec and 8 per sec it was driven when the rate of stimulation was increased to 34 shocks per sec Since stimuli at 0.6 and 34 shocks per sec were the extremes of the frequency range used in these experiments the possibility exists that the two upper curves of Fig 9 *A* might have shown also a second peak of responsiveness had rates higher than 34 shocks per sec been applied

Discussion

The demonstration in the present paper that intercostal-to-phrenic reflexes can be elicited merely by a contraction of the diaphragm itself has lent support to the idea that these reflex interconnections may be of physiological significance in making up, as it were, for the absence of autogenetic facilitation of the diaphragm The each

dorsal root discharge in response to a jerk of the diaphragm engaged also groups Ib and II afferents which, in the previous paper (Decima *et al.* 1968), were shown to be responsible for the intercostal-to-phrenic reflex. The latent period between the early dorsal root volley and the phrenic response also fits precisely the latency for the intercostal-to-phrenic reflex reported in the same paper. There can thus be little doubt that this early afferent volley is responsible for the short latency phrenic response to contraction of the diaphragm.

For the reasons given below it seems highly unlikely that the early afferent volley could be of an 'ephaptic' nature, i.e. caused by 'stimulation of the peripheral nerve terminals by active muscle' as observed in the ankle extensors of cat by Lloyd (1942) and later by Leksell (1945) and by Granit, Pompeiano and Waltman (1959). Firstly, the directly elicited diaphragmatic action current was hardly as synchronized as it has to be in order to stimulate peripheral nerve terminals (*cf.* Granit *et al.* on the difference in this respect between the ankle extensors and flexors). Secondly, the early volley and the phrenic response did not depend on whether the diaphragm had a high position with broad contact with the intercostal muscle or whether the lungs were inflated to give the diaphragm a lower initial position with a minimum of contact with the thoracic wall. Thirdly, the early afferent volley did not follow frequencies higher than 10 to 20 shocks per sec while spikes of 'ephaptic' origin in the ankle extensors follow frequencies of 100 to 200 per sec in slack muscle (Lloyd 1942, Granit *et al.* 1959). It thus seems fairly justified to exclude an 'ephaptic' origin of the early afferent discharge which is held responsible for the phrenic response. It can further be concluded that the self facilitation of the phrenic motoneurone pool is produced by the mechanical action of the diaphragm on the trunk wall and that the intercostal-to-phrenic reflex is a link in the phrenic self facilitation.

Tetanic stimulation of the peripheral end of the phrenic nerve produced not only an initial response but also a prolonged asynchronous discharge indicating that a sudden synchronous jerk by the diaphragm is not a prerequisite for the phrenic self facilitation, although its initial phasic component is stronger than the response to sustained contraction. The resemblance in this respect with the phrenic responses to mechanical stimuli applied directly to the trunk wall is obvious.

The second, third and sometimes fourth and fifth phrenic volleys appearing in response to a single diaphragmatic jerk were always abolished by a paralyzing dose of Flaxedil and were thus not due to intrinsic spinal mechanisms. These later waves indicate that phasic responses are fairly easily provoked and have a tendency of being self maintained provided the peripheral links through the muscles are intact. This synchronical reverberating activity may be looked upon as an exaggeration by the experimental conditions of more physiological mechanisms as clonus is of postural mechanisms.

The study of the excitability changes of the phrenic motoneurones has shown that the phrenic motor nucleus can hardly be regarded as a homogenous motoneuron pool with the uniform task of merely relaying the respiratory drive from the r

tory mechanisms in the medulla to the diaphragm Already the fact that the moto-units of the diaphragm are relatively small, with a muscle fibre to nerve fibre ratio comparable to that of the eye muscles (Krnjević and Miledi 1958), would suggest that the phrenic motoneurone pool may be highly differentiated

In these reflexes the order of recruitment has always been the same the small spike, indicating small diameter axons and probably small soma sizes, appeared before the bigger spikes and showed a higher probability of firing than the latter ones This is analogous with the more extensively studied organization of the lumbo-sacral motoneurones would seem to be at some variance with the conclusions of Gill and Kuno (1963) who did not find evidence justifying a subdivision of the phrenic motoneurones in tonic and phasic ones in analogy with the lumbo-sacral motoneurones The results of the present paper are however, in agreement with the recent work of Yaşargil (1967) who showed that this order of recruitment applied also to the respiratory drive of the phrenic motoneurones as suggested previously for the intercostal motoneurones (Corda *et al* 1965)

In the present paper the complexity of the organization of the phrenic motoneurone pool is suggested by the differencies in firing probability in response to variations of the three parameters stimulus strength, stimulus frequency and the nerve stimulated This has prompted further studies on cerebellar influences on the phrenic motoneurones and on possible topographical relations between the main portions of the diaphragm and the lower intercostal muscles This will be dealt with in a subsequent paper

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Descending Monosynaptic and Reflex Control of γ-Motoneurones

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Abstract

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The supraspinal and reflex control of γ motoneurones has been studied with intra and extracellular recording from lumbosacral γ-motoneurones in the cat Monosynaptic EPSPs were recorded in some γ-motoneurones on stimulation of the brain stem These effects were evoked from the Deiters' nucleus and from fibres descending in the medial longitudinal fascicle probably originating in the pontine reticular formation Previous investigations have revealed monosynaptic connections to α-motoneurones from these regions and our results suggest parallel effects to α- and part of the γ-motoneurone population supplying one muscle Indirect evidence suggests that this monosynaptic effect is exerted only on static γ motoneurones thus implying a linkage between the descending monosynaptic control of α and static γ-motoneurones via these pathways The reflex effects to γ-motoneurones have been studied with graded electrical stimulation of ipsi and contralateral hindlimb nerves The γ-motoneurones, presumably all belonging to extensor motor nuclei were found to receive IPSPs from group I afferents and it is suggested that only static γ-motoneurones are influenced. It has not been possible to decide if these IPSPs are evoked from Ib or Ia afferents. The reflex effects from group II and III muscular afferents joint and cutaneous afferents seem to conform to the effects evoked in α motoneurones from these afferents

After the demonstration of a special motor system to the muscle spindles (Leksell 1945, Kuffler Hunt and Quilliam 1951) it was soon found that γ-motoneurones could be influenced from different supraspinal regions (Granit and Kaada 1952 Granit and Holmgren 1955) but also from cutaneous and high threshold muscular afferents (Hunt 1951 Kobayashi Oshima and Tasaki 1952 Hunt and Paintal 1958) There is still no conclusive evidence regarding the important problem of whether γ-motoneurones receive autogenetic inhibition from group I afferents or not (Matthews 1964), although it has been suggested (*cf* Eldred Granit and Merton 1953, Hunt 1951 Brown Lawrence and Matthews 1968) One of the reasons why progress in the field of central control of γ-motoneurones has been rather slow is the difficulty in obtaining intracellular records from γ-motoneurones This technique has only been employed in one previous investigation (Eccles Eccles, Iggo and Lundberg 1960)

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The present investigation is largely concerned with the supraspinal control of γ motoneurones from two regions in the lower brain stem the Deiters' nucleus and the medial longitudinal fascicle (MLF) These regions are particularly interesting since stimulation of them yield monosynaptic EPSPs in α motoneurones to extensors (Lund and Pompeiano 1965, 1968, Grillner, Hongo and Lund 1966a 1968a) and flexors (Grillner and Lund 1966, 1968) respectively However, some results concerning the reflex control of γ motoneurones from spinal afferents will also be reported The effects that can be evoked from group I and other muscular afferents as well as cutaneous and joint afferents have been studied with graded stimulation of peripheral nerves in γ motoneurones recorded intra or extracellularly Some of the results have been reported in a preliminary paper (Grillner Hongo and Lund 1966b)

Methods

Preparation Experiments were performed on 15 cats operated under ether anesthesia which was followed [illegible]

[illegible] lateral funicle (*i e* ventral and lateral funicles) contralateral to the recording side was transected and separated from the ipsilateral for two segments which allowed the caudal part to be mounted on stimulated electrodes On the ipsilateral left side, usually a lesion was made leaving only the ventral quadrant intact and a stimulation electrode was placed on the medial surface of the ventral funicle rostral to the lesion In the hindlimb most muscle skin and joint nerves were dissected and mounted for stimulation on the left side and a few on the right (details for the particular nerves are found under Abbreviations) The skin flaps around the laminectomies and the dissected hindlimb nerves were sewn up to form pools which were filled with liquid paraffin

After removing the bone just caudal to the tentorium cerebelli thin tungsten electrodes insulated except for the tip (resistance 25—100 kΩ) were inserted in the brain stem by means of a Horsley Clarke apparatus The target structures were the ipsilateral Deiters nucleus and the ipsilateral medial longitudinal fascicle (MLF) at a level of approximately 5 mm rostral to obex To ensure that the correct electrode positions were obtained the Deiters nucleus was checked first by recording the antidromic field potential of the nucleus on stimulation of the vestibulospinal tract at lower thoracic level (Ito *et al* 1964 Grillner Hongo and Lund [illegible]
fast descending [illegible]
same brain stem [illegible]
method A fast [illegible]
very limited regi[illegible]
electrolytic lesion [illegible]
checked histologically

After the operation the cats were immobilized with gallamine triethiodide (Flaxedil® May and Baker Ltd) and artificially respired with a mixture of 6 % CO_2 in O_2 The arterial blood pressure was recorded continuously Blood loss was always substituted by a mixture of high and low molecular dextran The temperature in rectum and the paraffin pools was kept within 37—38° C

Stimulation and recording Square wave pulses of 0 2 msec durations were applied to the brain stem (between the tungsten electrodes cathode and an indifferent electrode in the neck muscles) All other stimuli used were condenser discharges with a half decay of 45 μsec Recordings were made with glass capillary microelectrodes filled with 3 M KCl or 2 M K-citrate Descending volleys and volleys in the dorsal roots were recorded from the cord dorsum by a ball tipped electrode at the L7 dorsal root entry zone against an indifferent electrode in the back muscles

Identification of γ-motoneurones The γ motoneurones were recorded intracellularly when possible and otherwise extracellularly

With intracellular recording the neurones were easily identified by the antidromic [illegible] from the muscle nerve in question without graded prepotentials and the laten[illegible]

formation about whether the conduction velocity was in the γ range (20—45 m/s) or not (cf Eccles et al 1960) However since it is known that there exist motoneurones that activate extrafusal muscle fibres and conduct with a velocity down to 31 m/s (Bessou Emonet-Dénand and Laporte 1965) the reflex effects were also checked. The lack of monosynaptic excitation from group Ia afferents to γ motoneurones is known from the investigation of Hunt and Paintal (1958) Eccles et al (1960) and Voorhoeve and van Kanten (1962) α motoneurones always receive Ia excitation. ABSm α motoneurones are sometimes poorly excited homonymously but almost invariably receive Ia excitation heteronymously (Eccles et al 1957a Lundberg personal communication) Moreover there is a positive correlation between the amplitude of the monosynaptic EPSP and the input resistance of α motoneurones (Burke 1968) i.e. a cell with a slowly conducting fibre would be expected to have a relatively larger EPSP

In four cases the ventral roots were cut and stimulation of the roots identified the neurones as motoneurones. The threshold of the axon involved relative to that of α fibres indirectly provided some information about the size of the fibre (Leksell 1945) In those cases the lack of Ia excitation was very cautiously investigated by stimulation of the sciatic nerve and by stimulation of the intact dorsal roots from L6 to S2

Since graded prepotentials are difficult to recognize in extracellular recordings the occurrence of a spike at a fixed and stable latency, of a value compatible with γ velocity on stimulation of one muscle nerve together with a threshold several times above Ia threshold was taken to indicate that the neurone was antidromically activated from a fibre conducting in the γ range and thus a γ motoneurone and not an orthodromically activated interneurone (cf Eccles et al 1960) The neurones that behaved in this fashion were located in the appropriate motor nucleus (Eccles et al 1960, Nyberg Hansen 1965) and were never monosynaptically activated from group I afferents on stimulation of any muscle nerve If a neurone of the above type was first recorded extracellularly and later impaled, it was always found to be a γ motoneurone by the above stated criteria

Abbreviations ipsilateral i contralateral co, Deiters' nucleus ND medial longitudinal fascicle MLF ventral quadrant of the spinal cord VQ ventrolateral funicle (i.e. the ventral and lateral funicles) VLF, flexor reflex afferents (i.e. group II and III muscular afferents cutaneous and joint afferents FRA postsynaptic potential PSP excitatory postsynaptic potential EPSP inhibitory postsynaptic potential IPSP, threshold thr millivolt mV millisecond msec milliampere mA nanoampere nA.

Quadriceps nerve Q posterior biceps-semitendinosus PBSt anterior biceps-semimembranosus ABSm suralis Sur hamstring nerves, Ham superficial peroneal nerve without muscle branches SP deep peroneal nerve without cutaneous branch DP gastrocnemius-soleus GS plantaris Pl flexor digitorum longus with flexor hallucis longus posterior tibial and the popliteus nerve without interosseus FDL posterior joint nerve to the knee J tibial nerve without branches to the crural muscles Tib

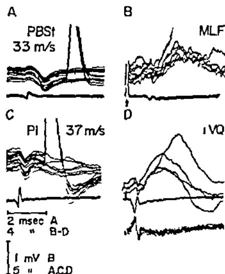
Results

1 *Descending monosynaptic effects to γ motoneurones*

Fig 1 shows intracellular records from a plantaris (Pl and a posterior biceps-semitendinosus PBSt motoneurone They are identified as γ motoneurones by the antidromic activation from the muscle nerve (A and C and also by the lack of Ia EPSP (cf Methods In D the ipsilateral ventral quadrant iVQ of the spinal cord is stimulated at the lower thoracic level An EPSP was generated in the cell with a segmental latency of 0 75 msec In this and other places the word "segmental latency will be used to indicate the interval between the peak of the cord dorsum positivity recorded at the segmental level and the start of the PSP cf Lund and Pompeiano 1968 In B an EPSP is generated in the PBSt cell with a segmental latency of 0 85 msec after stimulation of the lower brain stem in the region of the medial longitudinal fascicle MLF 5 mm rostral to obex Since the peak of positivity signals the arrival of activity in the vast majority of the fastest descending fibres (Lund and Pompeiano 1968 Grillner Hongo and Lund 1968b and the segmental latency measured from this point is well below 1 0 msec this suggests a mono-

Fig 1 Monosynaptic excitation of a flexor and an extensor γ motoneurone from descending fibres Upper traces illustrate intracellular records from a PBSt (A, B) and a Pl (C, D) γ motoneurone obtained with citrate electrodes The lower traces are from the dorsal root entry zone of L7 The lowermost record in D is recorded with the microelectrode tip at a position just outside the Pl cell Time and voltage calibrations as indicated the latter referring to upper traces only The lowermost traces in D are at higher amplification (twice) compared to the upper beam Only the ipsilateral ventral quadrant of the spinal cord was intact

In all illustrations (Fig 1—9) positivity is recorded upwards in the microelectrode recordings and downwards in the cord dorsum leads All structures stimulated are indicated by the appropriate abbreviation The figures in the antidromic records in this and the following illustrations indicate the axonal conduction velocity of each neurone in metres per second



synaptic linkage from descending fibres in the spinal cord to γ motoneurones belonging both to flexor and extensor motor nuclei Not all γ-motoneurones were excited from descending fibres with short latencies In Fig 6 for example, a PBSt γ motoneurone received powerful excitation from peripheral nerves (D L), while no trace of excitation could be evoked from either the ipsilateral or the contralateral thoracic cord (B, C)

Fig 2 shows intracellular records from a γ-motoneurone in upper L7 antidromically invaded from the ventral root at a stimulation strength of 2 1 times threshold for α fibres (A) This neurone does not receive monosynaptic excitation from any of the dorsal roots (B) from L6 to S2 nor from the sciatic trunk (C) or any peripheral nerve It is located in or very close to the ABSm motor nucleus

Stimulation of the iVQ (D) gives rise to an EPSP with a segmental latency of 0 4 msec and an amplitude of 7 0 mV This short latency virtually proves that the neurone is monosynaptically excited from descending fibres

In F-J the MLF is stimulated with increasing strength Already at 0 01 mA an EPSP is evoked (F) with a segmental latency of 0 8 msec at 0 03 mA there is an additional EPSP (G) with a shorter latency (0 45 msec) It is easy to distinguish two components of the EPSP and it seems as if the original EPSP (F) is superimposed on a smaller but earlier EPSP A slight increase of the stimulation strength yields a larger EPSP which is maximal at 0 08 mA (I) and does not become significantly greater on increasing stimulation strength to 30 times the threshold (J) The amplitude of the maximal EPSP (J) is of equal size to the one obtained by a supramaximal stimulation of the ipsilateral cord (D) which indicates that the fibres that give rise to this EPSP are all of supraspinal origin

A stimulation with an electrode carefully located in the Deiters nucleus (K M) did not have any effect at a strength of 0 15 mA but had some at 0

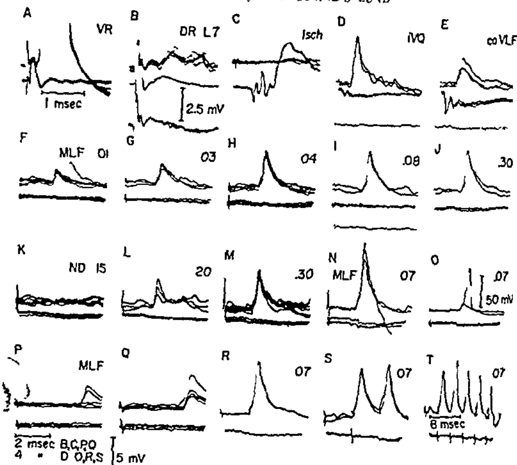


Fig 2 Descending monosynaptic control of a γ motoneurone from MLF The upper traces are intracellularly recorded with a citrate electrode The lower ones record the descending (or afferent incoming) volley at the level of the dorsal root entry zone of L7 The bottom traces in B D E and J are extracellularly recorded at a position just outside the cell Stimulation strength is expressed in mA Time and voltage calibrations as indicated the latter referring only to the upper beam Note particular insets in A B and O The neurone is identified as a γ motoneurone by *the antidromic* activation from the ventral root (A) together with the lack of monosynaptic excitation from any peripheral nerve or any dorsal root from L6 to S1 (*cf* B C) The neurone was *recorded* in the vicinity of the ABSm nucleus N and O were recorded after tetanic stimulation of MLF Only the ipsilateral ventral quadrant of the spinal cord was intact

yielded a maximal EPSP (compared to J) at 0.30 mA The threshold is thus 15–20 times higher than from MLF Hence it seems likely that fibres in the region of the Deiters' nucleus do not contribute to the EPSP and that the effects are evoked by spread to other structures In records N and O is shown that stimulation of the MLF can give rise to an EPSP that triggers a spike of an amplitude of 67 mV in this case These records were obtained after high frequency stimulation of the MLF, possibly causing postactivation potentiation In P MLF is stimulated at threshold and an EPSP of 2.2 mV can be seen to occur in an all-or-none fashion indicating that this EPSP can be due to a liminal stimulation of a single descending

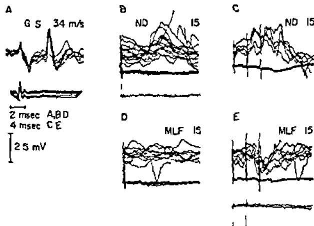


Fig 3 Excitation and inhibition evoked in a γ motoneurone from the brain stem The upper traces show intracellular records from a G-S γ motoneurone with a citrate electrode The lower traces are from the dorsal root entry zone of L7 The lowermost traces in B and E are recorded with the microelectrode tip at a position just outside the cell Time and voltage calibrations as indicated, the latter referring only to microelectrode recordings Only the ipsilateral ventral quadrant of the spinal cord was intact On stimulation (0 15 mA) in the region of the Deiters nucleus a monosynaptic EPSP is evoked and from MLF an IPSP

axon from the brain stem and thus is unitary With a slight increase of the stimulation strength, the former EPSP always occurs (Q), but in one trace an earlier EPSP also appears The difference in latency between the earlier and the later EPSP is 0 35 msec The early EPSP can be seen in all records at a stimulation at or above 0 03 mA (MLF) This difference is not due to an additional synapse at the segmental level since the 'early' and the 'late' EPSP can also be distinguished on stimulation of the iVQ (D) but the interval is now only 0 15 msec, which means that it is due to different conduction velocites in the descending axons generating the EPSP The entire EPSP in R seems to be monosynaptic since it does not change appreciably in shape on repetitive stimulation in S or T (300 c/s) and the different components are virtually unchanged

Stimulation of the contralateral ventrolateral funicle (coVLF) at the lower thoracic level yields an EPSP (E) with a segmental latency of 0 9 msec This value indicates that the linkage is monosynaptic especially since the fibres from the contralateral side may have to cross in the segment and thus have a longer conduction distance On only two occasions excitation was demonstrated from the coVLF with a latency indicating a possible monosynaptic linkage Further studies are needed to determine if this short latency excitation is of common occurrence Monosynaptic excitation also occurs in *α* motoneurones from descending fibres in the coVLF which was described by Willis *et al* (1967) and which has recently been confirmed in this laboratory (Bruggencate Burke and Lundberg unpublished)

Fig 3 shows intracellular records from a G S γ motoneurone Stimulation of the region of the Deiters nucleus (0 15 mA) yields an EPSP with a segmental latency of 0 8 msec (B) No postsynaptic effect is evoked on single stimulation from the MLF (D) but with three shocks an IPSP (E) Thus there are reciprocal effects evoked from the two different regions in the same extensor γ motoneurone This reciprocal pattern from the two regions can be evoked in *α* motoneurones to extensors (Grillner Hongo and Lund 1968a) This is the only γ motoneurone in w

this reciprocity was demonstrated (Fig. 3). However, inhibitory effects could not be investigated in the larger part of the material which was obtained by extracellular recording.

Fig. 5 shows intracellular records from a plantaris γ-motoneurone with a chloride electrode. The hyperpolarizing IPSP initially elicited from the periphery (Q, R) quickly reversed (I, L) presumably due to diffusion of the chloride ions from the tip of the microelectrode (Coombs, Eccles and Fatt 1955). Stimulation of the i. VQ yields a synaptic potential with a segmental latency of 0.7 msec (C) but no effects from the contralateral side (D). Stimulation in the region of Deiters' nucleus produced a PSP with a segmental latency of 0.6 msec (B). The stimulus was just at threshold for the EPSP and in two traces a synaptic potential of a similar shape was evoked with a 1.1 msec longer latency. Since the shape was similar it is possible that the later potential was mediated by the same descending fibres as the "early" and that the descending neurones were activated synaptically in the brain stem instead of directly by current. Stimulation of this strength (0.15 mA) did not give monosynaptic excitation in α-motoneurones to flexors although this could be obtained from the MLF and thus current spread to the medial region is not likely. It is not possible from these records to differentiate between EPSP and IPSP, but since there is no clear evidence from either γ-motoneurones or α-motoneurones that there exists a monosynaptic inhibitory descending pathway from this region (cf. Grillner et al. 1968b) conducting at a speed above 100 m/sec it is reasonable to assume that the PSP elicited from the brain stem is an EPSP.

Our material includes 12 γ-motoneurones recorded intracellularly, in 8 of which excitation was evoked from descending fibres with a segmental latency of 0.4–0.9 msec, in one neurone at 1.2 msec and in three there were no appreciable monosynaptic EPSP evoked by a descending volley (cf. Table I).

The extracellular material consists of 28 γ-motoneurones, 17 of which were activated by descending fibres stimulated either at thoracic cord level or in the brain stem. When single stimuli were not effective in evoking a spike discharge, from two to five shocks were employed to cause temporal summation of the descending effects. The shortest segmental latency for the effective volley to elicit discharges was measured and is classified in four groups as in Table I. Nine cells were excited with a latency below 1.25 msec (Table I). In this segmental latency the initiation time of the spike is included. In the cells intracellularly recorded, where the EPSP evoked from descending fibres triggered a spike, the time from the onset of the EPSP to the onset of spike was minimally 0.3 msec (cf. Fig. 2 N, O). This means that when estimating the latency for the EPSPs at least 0.3 msec shall be subtracted from the values given in Table I and it seems justified to conclude that the group of nine γ-motoneurones

Table I

Intracellular recording				
Segmental latency in msec	0.4–0.9	1.2	no actual	
Number of γ-motoneurones	8	1	3	
Extracellular recording				
Segmental latency in msec	0–1.2	1.26–2.0	2.1–4.0	4.0–
Number of γ-motoneurones	9	5	4	10

fired with latencies shorter than 1 25 msec are monosynaptically activated from descending fibres The second group of cells that are excited with latencies between 1 25—2 0 msec can be activated through descending polysynaptic or monosynaptic connexions It is not possible to decide between those alternatives since the initiation time can probably vary considerably The group of γ motoneurones that were not excited with short latencies (the last group in Table I) might receive either synaptic effects subthreshold to evoke a spike or no effects at all

In summary, our results indicate that out of 40 γ motoneurones recorded from, extra or intracellularly, monosynaptic excitation could be evoked from descending fibres in 16, while 10 were excited with longer latencies In the residuum it was not possible to reveal any effect In 11 cells it was shown that the monosynaptic effects originated from the brain stem, and though the effects could sometimes be elicited from both regions investigated (*cf* Fig 2), the threshold from one was always lower No detailed analysis concerning the exact location of the two regions was carried out (*cf* Grillner *et al* 1968), since it was usually only possible to record from a γ motoneurone for a very short time However α- and γ motoneurones of the same motor nucleus have been found to receive monosynaptic EPSPs from the same region *i e* either the Deiters nucleus or the MLF The possibility still remains that the effects are due to other fibres passing through or in the close vicinity of the Deiters' nucleus and from another fibre system within the MLF, respectively Another possibility is that the effects are due to antidromic activation of large ascending fibres which could activate the motoneurones by collaterals at the spinal level This is, however, very improbable since collaterals of this type have not been described and since it is known from lesion experiments that at least α motoneurones receive monosynaptic excitation only from descending fibres (Lund and Pompeiano 1968)

2 *Postsynaptic effects from primary afferents*

a *Group I afferents*

Fig 4 shows a γ motoneurone located in the G S motor nucleus (for identification *cf* methods) Weak stimulation of the G S nerve yields an IPSP in G that commences at the time indicated by an arrow The preceding negativity is due to the extracellular field (lowermost trace) caused by the Ia volley This IPSP increases slightly with higher stimulation strength (H I) but judged from the amplitude of the incoming volley (middle trace) the group I component is far from maximal in G The IPSP is approximately maximal in H and does not grow further at higher stimulation strength (8 22×thr in J) although a later IPSP is added These results indicate that the IPSP is evoked from group I afferents Stimulation of the plantaris nerve yields an IPSP in L and M in which the stimuli are below the maximal group I strength (*cf* the incoming volley recorded in lower or middle traces) T IPSP (M) does not change considerably at higher stimulation strength (N) inhibition is evoked also by a plantaris group I volley On stimulation of an EPSP is evoked with a latency of 0 9 msec (B) suggesting a n

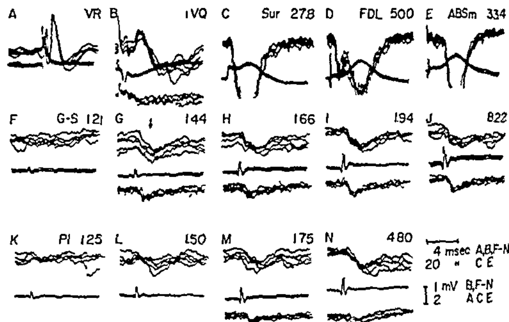


Fig 4 Inhibition from group I afferents in a γ motoneurone The upper beams show intracellular records with a citrate electrode from a cell antidromically invaded from the ventral root (A) and with no monosynaptic excitation from any peripheral nerve or dorsal root The lower beams are from the dorsal root entry zone of L7 The lowermost records in B G J M and N are recorded with the microelectrode tip just outside the cell Time and voltage calibrations as indicated the latter referring only to microelectrode traces Only the ipsilateral ventral quadrant of the spinal cord was intact On stimulation of peripheral nerves the stimulation strength will in this and consecutive figures be given in multiples of the threshold for the most excitable fibres in the nerve as judged by the afferent volley recorded at the dorsal root entry zone

linkage A very strong inhibitory action is evoked from cutaneous and high threshold muscular afferents (C E)

In Fig 5 reflex effects to a plantaris γ motoneurone recorded with a chloride electrode is shown The neurone is antidromically invaded from the muscle nerve (A, Q) (28 m/s) and receives no monosynaptic excitation from group I afferents Stimulation of the G S nerve (E-H) at increasing strength from 1 08 times threshold of group I (E) to 2 17 times threshold i e just above group I maximum (H) yields synaptic potentials At least in two traces in E (1 08 × thr) PSPs are evoked at the same latency as in F Hence it can be concluded that synaptic effects are evoked from group I afferents Increasing stimulation strength yielded larger synaptic potentials In I L the autogenetic effects from group I afferents are illustrated The effects from another muscle nerve FDL are given in M P showing that the threshold for synaptic actions is below 1 37 times threshold (M) and the synaptic potentials do not seem to increase between 2 45 (O) and 2 81 times threshold (P)

In Q and R the plantaris nerve is stimulated near the threshold for the axon of the γ motoneurone (Q) directly after impalement of the cell If the extracellular field is subtracted it is seen that there is an IPSP evoked in the cell with the same

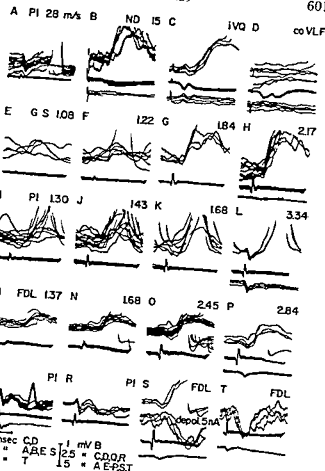


Fig 5 Postsynaptic potentials evoked from group I afferents in an extensor γ motoneurone The upper traces are intracellular records from a γ motoneurone obtained with a chloride electrode The lower traces are from the dorsal root entry zone of L7 and the lowermost from a microelectrode position just outside the cell Time and voltage calibrations as indicated the latter referring only to microelectrode records In Q the extracellular record is taken at half the amplification of the corresponding intracelullar Only the ipsilateral ventral quadrant of the spinal cord was intact Q and R are taken just after impalement of the cell before the IPSPs have been reversed (I L) by chloride diffusing from the tip of the electrode A depolarizing current of 5 nA was passed during upper beam in T and the second beam in S

latency as the depolarizing response of records I L which was taken some minutes later A reversal of the IPSP was expected to occur due to a chloride leakage into the cell It is thus very likely that there is an autogenetic inhibition evoked in this γ motoneurone from group I afferents (*cf* I L) In S upper beam the nerve to FDL is stimulated at 6 65 times threshold and in the middle beam a depolarizing current of 5 nA is passed during the same nerve stimulation This reverses the PSP to a hyperpolarizing response It is seen that the hyperpolarizing response has the same latency and a similar shape as the depolarizing (S T) This makes it likely that the first part of the synaptic potential evoked from FDL is a reversed IPSP and not an EPSP which probably also is the case for the effects from G S In T the full time course of the hyperpolarizing response in S is shown on a slower time base

The latency of the PSPs have generally been found to decrease on increasing stimulation strength within the group I range G S stimulation yields latencies from 2 6 to 1 8 msec and FDL and Pl from above 3 msec to 2 6 msec The shorter latency (1 8 msec) is suggestive of a disynaptic linkage and the longer of a trisynaptic The

shortening probably also reveals a more efficient activation of the interneurones mediating the effect. The central latencies are comparable to those obtained from group Ib afferents to α motoneurones to which there are both di- and trisynaptic pathways (Eccles *et al.* 1957b)

Five γ motoneurones have been analyzed with graded stimulation of muscular afferents. In these neurones postsynaptic effects could be evoked at group I strength. These γ motoneurones all probably belonged to extensor motor nuclei since two were identified by antidromic activation from the muscle nerve (G-S and Pl) and the other three although not identified from the muscle nerve were found within the G-S (*cf* the large monosynaptic extracellular negativity in Fig 4) the plantaris and in or very close to the ABSm (*cf* Fig. 2) motor nucleus respectively. Four of the neurones were tested for descending effects (Fig 2 4 5) and were found to receive monosynaptic excitation.

b Cutaneous joint and high threshold muscular afferents

In this series of experiments preparations with the ipsilateral ventral quadrant of the spinal cord intact were generally used although in one case a spinal animal was employed. It is known that several descending pathways influence transmission in spinal reflex arcs (*cf* Lundberg 1966) and that they can profoundly change the spinal reflex pattern. An intact preparation might well reveal different reflex connexions compared to a spinal. However since the dorsal parts of the lateral funicle ... cut, the descending systems that mediate tonic inhibitory control of reflex actions from the FRA (group II group III muscular afferents and cutaneous and joint afferents Holmqvist and Lundberg 1961 were removed together with the rubrospinal and the pyramidal tracts which both have facilitatory effects on interneurones in reflex arcs Lundberg Norrsell and Voorhoeve 1962 Hongo Jankowska and Lundberg 1965

Fig 6 shows an intracellular recording with a citrate electrode from a FBst γ motoneurone (A Stimulation of the G S nerve gives repetitive activation of the neurone (D All nerves that are stimulated in E-L also generated spikes (SP not tested) To be able to study the excitation without spike generation a hyperpolarizing current of 10 nA was passed *cf* D and K EPSPs are evoked from cutaneous (E-G) joint (H and muscular afferents from hip knee and ankle extensors as well as ankle flexors I L In this particular case there is mostly excitation but concealed inhibition cannot be excluded. The shortest latency is 1.85 msec for the EPSP elicited from the sural nerve. This is in agreement with the central delay of 2.03—2.46 msec that Hunt and Paintal 1958 calculated after compensating for conduction time in the axon. Since they are calculating the time to onset of the spike our values of central delay of the EPSP expectedly would be somewhat shorter. Eccles and Lundberg 1959 originally suggested that the excitation from flexor reflex pathways to alpha motoneurones is disynaptic and the short central latency could be compatible with such a linkage. However it has since been postulated that the corresponding pathway to alpha motoneurones is trisynaptic (Lundberg 1958

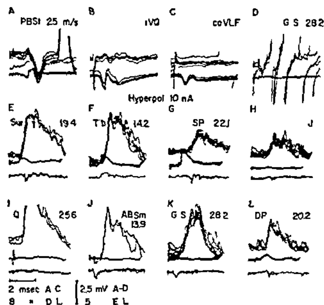


Fig 6 Excitation of a flexor γ motoneurone from FRA The upper traces show intracellular records from a PBSt γ motoneurone with a citrate electrode The lower or middle (E-L) traces are recorded from the dorsal root entry zone of L7 The lowermost traces in E-L show the extracellular fields just outside the cell In E-L a hyperpolarizing current of 10 nA was passed through the microelectrode Time and voltage calibration as indicated the latter referring only to microelectrode recordings Only the ipsilateral ventral quadrant of the spinal cord was intact All nerves stimulated in E-L generated one or more spikes when no current was passed through the microelectrode (SP not tested)

When considering the above latency of 1 85 msec it should be noted that it represents an extreme (usually this latency was 2—3 msec) which may be compatible with a trisynaptic linkage

The reflex excitation in which trains of spikes (or large EPSPs as in Fig 6) were evoked to another in which virtually no excitation could be evoked from the peripheral nerves Hence our findings are in agreement with those of Hunt and Paintal (1958) Unfortunately we have not obtained intracellular records from a flexor γ motoneurone with no reflex activation

Fig 7 illustrates intracellular records from an extensor (G S) γ motoneurone (A) The ipsilateral ventral quadrant was intact No certain monosynaptic EPSP can be evoked on stimulation of the region of the Deiters nucleus (C) or the NQ (B) but descending inhibition is obtained in B The cell was probably at a rather depolarized level which influences the size of the IPSP Thus the absolute values might not be comparable to values under physiological circumstances Inhibition is evoked from joint (D) and cutaneous afferents (G H K L N) as well as from nerves to knee and toe extensors and to ankle flexors (E F I J M) (cf also Fig 4 C E) Some excitatory effects might be concealed by the large IPSPs The latency of the IPSP from the superficial peroneal nerve is 2 4 msec from the mixed tibial nerve 1 95 msec and from FDL 1 7 msec The inhibitory action from FDL and the tibial nerve can be evoked either from group I afferents or FRA The latency of 1 7 msec is so short that it is hardly compatible with a trisynaptic linkage In contrast the shortest latency from cutaneous afferents is 2 4 msec and suggests that there is a trisynaptic linkage from those afferents (Eccles et al 1957b Eccles and Lundberg 1959) From contralateral nerves (O P) no strong synaptic action is evident

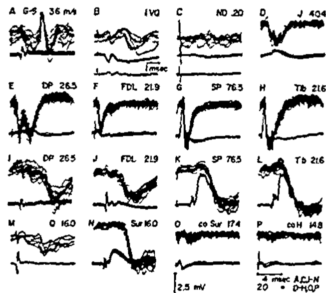


Fig 7 Inhibition of an extensor γ motoneurone from FRA The upper traces show intracellular records from a G S γ motoneurone with a citrate electrode The lower traces are from the dorsal root entry zone of L7 The lowermost traces in B and C show the extracellular records at a position just outside the cell Time and voltage calibrations as indicated the latter referring only to the upper beam Note particular inset in B Corresponding records in E H and I L respectively are taken simultaneously but with different time base In C stimulation strength is given in mA Only the ipsilateral ventral quadrant of the spinal cord was intact

Fig 8 is a schematical representation from an extracellular recording of a G S γ motoneurone in a spinal cat The neurone has a resting discharge (see Fig 8, resting) of about 20/sec Weak single stimuli to cutaneous afferents (SP Tib Sur) evoked an early inhibition followed by a late (45 msec after stimulation) excitation The conclusion that the neurone has a decreased excitability is based on the fact that in several records no spike occurs during an interval of 45 msec after the stimulation although the neurone has a resting discharge This inhibition can be caused either by a decrease of background impingement from interneurones mediating tonic excitation to the γ motoneurone or by a true postsynaptic inhibition With higher stimulation strength the excitatory phase vanished and an inhibition of longer duration replaced it The IPSPs recorded on stimulation of peripheral nerves in the cell of Fig 7 had durations up to approximately 25 msec

Eldred and Hagbarth (1954) found that G S γ motoneurones were accelerated on adequate stimulation of the skin covering gastrocnemius and its tendon which is supplied mainly by the sural nerve but to some extent also of SP and Tib When conditioning a monosynaptic reflex from the G S nerve with a sural nerve volley Hagbarth (1952) often found a decreased excitability for 25—35 msec followed by facilitation in the spinal preparation The present variation in excitability of the γ motoneurones on weak stimulation has a similar time course to the one described by Hagbarth (1952) for α motoneurones

Fig 9 shows extracellular records from another type of G S γ motoneurone in a spinal cat This neurone is fired repetitively by volleys in cutaneous afferents with a latency between 2 8 and 4 15 msec showing that extensor γ motoneurones also can receive short latency excitation from cutaneous afferents (B-D) It is also activated from some muscular afferents and from joint afferents although less effectively (E-F) It was recorded in the same preparation as the neurone in Fig 8 and just

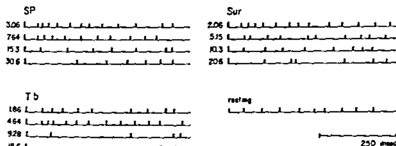


Fig 8 Reflex actions from cutaneous afferents to an extensor γ motoneurone This is a schematical representation of an extracellular recording from a G-S γ motoneurone in a spinal preparation [illegible]

left of each sweep

before the latter, although the short latency action is inhibition in Fig 8 and excitation in Fig 9

Of six G S γ motoneurones recorded in a spinal preparation most received excitation from the sural superficial peroneal and mixed tibial nerve with a central latency below 10 msec In preparations with intact ventral quadrant the corresponding finding was also frequent Extensor γ motoneurones were sometimes excited from ipsilateral muscular afferents with short latency

Discussion

1 *Descending monosynaptic effects to γ motoneurones*

The descending monosynaptic effects to γ motoneurones can be evoked at low threshold by stimulation of the same brain stem region from which monosynaptic excitation is evoked in α motoneurones (Lund and Pompeiano 1968 Grillner *et al* 1968a, Grillner and Lund 1968) In both cases the effects are mediated by fast conducting fibres located in the ipsilateral ventral quadrant of the spinal cord By analogy with the findings on α motoneurones it is postulated that the pathways with monosynaptic connexions to γ motoneurones originate in Deiters nucleus and the lower pontine reticular formation (Grillner and Lund 1968) respectively However

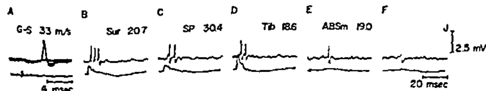


Fig 9 Excitation of an extensor γ motoneurone from FRA The upper traces show intracellular records with positive spike from a G-S γ motoneurone in a spinal preparation. The lower traces are from the dorsal root entry zone of L7 The voltage calibration refers only to upper beam The neurone could be activated from nerves indicated but also from a few other ipsilateral muscular nerves. The joint nerve (F) was stimulated at a strength including high threshold joint afferents

it may well turn out difficult to prove this postulate—experiments with chronic lesions which were very valuable in the analysis of effects in α motoneurones (Lund and Pompeiano 1968) cannot be used since the descending monosynaptic effect is lacking in one group of γ motoneurones (*cf* below) and only a very limited number of γ motoneurones can usually be recorded in each experiment

In most extensor motor nuclei monosynaptic EPSPs are evoked exclusively from Deiters nucleus in a few from MLF and in flexor motor nuclei from the latter region (Grillner *et al* 1968a) An important question is whether from the two brain stem regions there are parallel effects to α and γ motoneurones of the same muscle As has already been discussed under results, the present investigation suggests that this is the case but it should be recalled that the material is very small

α and γ motoneurones seem to be different with respect to the frequency of occurrence of descending excitation According to Lund and Pompeiano (1968) about 80 % of the α motoneurones receive descending monosynaptic excitation In the present material there was evidence for this descending effect only in 17 of 40 recorded γ motoneurones However this figure may be too low because in the 28 extracellularly recorded γ cells there is no certainty that the extracellular technique allowed detection of all cells with descending monosynaptic connexion even though repetitive stimulation was regularly employed (*cf* Table I) With intracellular recording on the other hand descending monosynaptic EPSPs were evoked in 8 of 12 γ motoneurones This percentage is only slightly lower than in α motoneurones Nevertheless we did in our preliminary report postulate that there are two functional classes of γ motoneurones one with and another without descending monosynaptic effects (Grillner *et al* 1966b) This postulate was based on the observation that the descending EPSP when occurring seems to be larger in γ than in α motoneurones (*cf* below) Hence it appeared less likely that the absence of effect represented one extreme within one functional group of cells In relation to our tentative suggestion (Grillner *et al* 1966b that the descending pathways have monosynaptic connexions with static but not with dynamic γ motoneurones it is noteworthy that the former are more common than the latter (Bessou and Laporte 1966) The above suggestion is strongly supported by recent findings by Bergmans and Grillner (1968a) On the basis of differences in spontaneous activity and reflex effects under a variety of conditions they were able to classify recorded flexor γ motoneurones as dynamic or static Evidence for descending monosynaptic excitatory action was obtained exclusively in γ motoneurones classified as static This strongly suggests that the effect evoked from the MLF is exerted only on static γ motoneurones

There is no direct evidence concerning the effects to extensor γ motoneurones but the striking similarities in functional organization of the descending effects to extensor and flexor motor nuclei Grillner *et al* 1968a by suggest that the monosynaptic effects to extensor γ motoneurones also might be exerted on only the static type Some support for this idea is given by Carli Diete-Spiff and Pompeiano (1967) On stimulation of the Deiters nucleus they recorded acceleration of afferents with secondary endings from extensors These afferents are only influenced by static

γ motoneurones (Appelberg Bessou and Laporte 1966 Brown Engberg and Matthews 1967) Thus it can be concluded that static γ motoneurones are excited from this region but a conclusion concerning the synaptic linkage causing this response in secondary endings cannot be drawn from their experiments

It is our impression that the descending monosynaptic EPSP is larger in than in α motoneurones In α motoneurones with resting membrane potentials of 60 to 70 mV the average value of the descending monosynaptic EPSP is 0 8 mV (Grillner *et al* 1968b) while the average value in the 8 γ motoneurones with descending monosynaptic EPSP was 3 1 mV, ranging between 0 5—9 mV (median value 2 0 mV) However most of these cells had low resting potential and hence reduced EPSPs The maximal amplitude of the descending monosynaptic EPSP was 9 mV Since this observation was made in an exceptionally well recorded cell with a spike potential of 67 mV it may well be more representative than the findings on cells with low membrane potential

It is interesting to note that the unitary EPSP (2 2 mV) of Fig 2 K is much larger than the estimated value for the unitary descending monosynaptic EPSP in α motoneurones (Grillner *et al* 1968b) This large value does however not necessarily imply a difference in synaptic organization of descending effects to α and motoneurones but may at least partly be explained by the assumption that γ motoneurones have smaller cell size than α motoneurones and thus higher input resistance The same quantal release giving rise to the same synaptic current will yield a larger PSP in a cell with a high input resistance as compared to one with a low (Katz and Thesleff 1957 Henneman *et al* 1965 Burke 1968)

The presence of monosynaptic connexions to both α and motoneurones raises the question of a possible α γ linkage This problem cannot be solved by an analysis of the present type since it is difficult to exclude the possibility that the synaptic connexions with α and γ motoneurones are made by functionally separate neurones However the similarity in organization of the descending effects to α and γ motoneurones is so striking that the linkage hypothesis seems more likely The possibility of an α γ linkage from these descending systems provide a challenge for further work for the particular reason that the connexions are monosynaptic It is of considerable interest that this assumed linkage seems to be between static motoneurones and α motoneurones to both slow and fast muscles A further implication of this linkage refers to the cerebellar control of movement via the vestibulospinal tract The large size of the EPSP that can occur in motoneurones Fig 2) indicates that even if the excitability is raised simultaneously in both α and motoneurones the latter might be fired in advance of the former The fact that the γ-discharge might be leading however cannot be taken to indicate that the loop (Eldred *et al* 1953) in this particular case is an alternative motor route in its own right (Granit 1966) It is difficult to correlate the present findings to the α and decerebrate rigidity described by Granit *et al* 1955) although it is known that the Deiters nucleus is of importance for the development of rigidity in the intercollicularly decerebrated animal (Sprague and Chambers 1953)

Granit and Holmgren (1955) could excite γ motoneurones with a latency of 7 msec from contralateral tegmentum at the level of the inferior colliculus The effects were mediated by fibres in the ipsilateral ventral funicle Thus there is a possibility that those effects are mediated by the same descending fibre system as in the present study and that the neurones responsible for the present excitatory effects are synaptically activated from the contralateral tegmentum

Granit, Pompeiano and Waltman (1959a) later showed that they could obtain acceleration of spindle afferents on stimulation of the Deiters' nucleus and the MLF, with such a short latency that they concluded that the spindle acceleration was caused by impulses in α fibres. *Their latencies are so short that γ-activation cannot account for the spindle activation even* through the fast conducting pathway investigated in the present work From experiments with nerve stimulation Granit, Pompeiano and Waltman (1959b) further suggested that the early spikes elicited at the "foot of contraction" were due to contraction of the intrafusal muscle fibres innervated by α fibres and that the supraspinal activation of spindle discharges with short latencies (Granit *et al* 1959a) was caused by the α innervation of muscle spindles. [illegible] onstrated that there are two [illegible] slowly conducting axons and [illegible] nother with fast conducting [illegible] the spindle afferents Since the former type did not give rise to a spike in a spindle afferent at the "foot of the contraction" and since the excitation of the spindle afferents that Granit *et al* (1959b) ascribed to α innervation seemed to be evoked from α fibres that had low threshold and were fast conducting, it is not possible to say with certainty if their "fast supraspinal control" (1959a) activates intrafusal muscle fibres in the muscle spindle by collaterals of α fibres or acts through mechanical interaction

Pompeiano and co-workers (Pompeiano et al 1967, Carli et al 1967) have demonstrated that repetitive stimulation of the Deiters' nucleus can give rise to [illegible] ion of spindle afferents also without extrafusal contraction This effect was [illegible] er early in onset and resistant to anesthesia. These facts led them to suggest that there is a monosynaptic linkage to γ-motoneurones from the Deiters nucleus. To determine the detailed intraspinal synaptic organization by recording the afferent *discharge from the muscle spindles is in our opinion virtually impossible*

Through the work of Andersson and Gernandt (1956) it is known that γ-efferents recorded in ventral root filaments are accelereated on vestibular nerve stimulation The large cells in the dorsal part of the Deiters nucleus do not have any connexion from vestibular primary afferents in contrast to the small cells located in the ventral part (Walberg Bowsher and Brodal 1958) Both project to the spinal cord For this and various other reasons Brodal (1967) suggested that *the small cells might be* responsible for the acceleration of γ-motoneurones and the large cells *of α motoneurones* However our results show that fast conducting *fibres in the region of the* Deiters' nucleus can cause monosynaptic excitation of lumbar γ motoneurones The large cells in the dorsal part of the Deiters nucleus are known to receive direct inhibitory control from the cerebellar Purkinje fibres (Ito and Kawai 1964 as well as excitation from collaterals of climbing fibres and other *cerebellar afferents* (Ito Obata and Ochi 1966 Ito and Yoshida 1966)

2 *Postsynaptic effects from primary afferents*

a *Group I afferents*

Autogenetic inhibition by muscle stretch was *reported to occur in* γ-efferents to medial gastrocnemius in the decerebrate preparation (Hunt 1951) Whether this

effect can be attributed to activation of group I afferents is not known since muscle stretch also activates spindle afferents with secondary endings that probably have an inhibitory action on extensor γ motoneurones (Voorhoeve and van Kanten 1962). Eldred *et al* (1953) reported autogenetic inhibition of γ motoneurones together with acceleration of a group Ib fibre on muscle stretch in the decerebrate preparation. Their conclusion that this inhibition is autogenetic has been critizised (Matthews 1964). Very recently Brown, Lawrence and Matthews (1968) reported that presumed γ efferents recorded in ventral root filaments were inhibited during selective activation of group Ia afferents by vibration (Brown, Engberg and Matthews 1967b). Since γ motoneurones can receive recurrent inhibition (Ellaway 1968, *cf* also Brown *et al* 1968), which at least partly *seems to be evoked from α fibres* (Brown, Lawrence and Matthews, personal communication), it cannot be excluded that these inhibitory effects are due to the concomitant very effective α acceleration that could depress the spontaneous activity in γ motoneurones through recurrent inhibition.

IPSPs were evoked at group I strength in five of the twelve intracellularly recorded γ motoneurones. The possibility that the inhibitory effects would be due to recurrent inhibition evoked from direct stimulation of α fibres can be excluded since inhibition at group I strength occurs also when the ventral roots are cut (Fig 4). Moreover, in analogy with α-motoneurones a recurrent IPSP would be expected to have a duration of 50 msec (Eccles, Fatt and Koketsu 1954). All five γ motoneurones probably belonged to extensors (*cf* Results) and the IPSPs were evoked from extensor nerves including the homonymous nerve. This pattern rather resembles that of Ib inhibition to α motoneurones. Inhibitory effects from Ib afferents to γ motoneurones would be an interesting possibility since such an effect could provide an explanation for the findings by Eldred *et al* (1953) (*cf* however Matthews 1964) and contribute to the understanding of the clasp knife phenomenon (Sherrington 1909). The latencies of the group I IPSPs in γ motoneurones are not of the same order as for the disynaptic Ia IPSPs in antagonist α motoneurones (Eccles *et al* 1956) but more resemble those found for group Ib IPSPs (Eccles *et al* 1957b). However, our results with graded stimulation of group I afferents do not allow a conclusion whether the inhibition is caused by activation of Ia or Ib afferents. The possibility that spindle afferents with primary endings may be responsible cannot be excluded (*cf* Brown *et al* 1968). The functional implication would also be a negative feed back of muscular activation.

The common method of revealing inhibition in γ motoneurones has been to observe a decrease in the resting discharge. It is interesting to note that in experiments with decerebrated cats where both static and dynamic γ motoneurones are tonically active (Jansen and Matthews 1962) autogenetic inhibition has been reported (Hunt 1951, Eldred *et al* 1953, Brown *et al* 1968). On the other hand in the spinal preparation where only dynamic γ motoneurones are spontaneously active (Alnaes, Jansen and Rudjord 1965, Bergmans and Grillner 1968b) attempts to demonstrate autogenetic inhibition have failed (Hunt and Paintal 1958). Evidence for in-

hibitory effects by electrical stimulation is very scanty (Eccles *et al* 1960 Voorhoeve and van Kanten 1962) These findings would be explained if the inhibition from group I afferents was only evoked in static γ motoneurones and in this connexion it is most relevant to note that the four γ motoneurones with group I IPSPs that were tested for descending effects received monosynaptic EPSPs (*cf* Bergmans and Grillner 1968a *cf* discussion page 15)

b *Cutaneous, joint and high threshold muscular afferents*

Hunt (1951) claimed that γ motoneurones followed the general flexion reflex pattern described by Sherrington (1910) In 1954 Eldred and Hagbarth demonstrated that γ motoneurones to some muscles were influenced by skin reflexes in the same way as α motoneurones (Hagbarth 1952) In a later study of single γ-efferents in peripheral nerve filaments Hunt and Paintal (1958) reported that there is a great variability in reflex excitability showing that some neurones with high spontaneous activity were efficiently activated reflexly others not spontaneously active were less readily excited and a few not excitable at all. On electrical stimulation of different peripheral nerves they concluded that it was not possible to find any meaningful pattern.

Subsequently it has been demonstrated that there are both excitatory and inhibitory pathways from FRA to flexor α motoneurones (Eccles and Lundberg [illegible]) which can be separately controlled from descending pathways (Holmqvist and Lundberg 1961 Both reflex pathways can sometimes be revealed in the spinal preparation Eccles and Lundberg 1959 When taking this into consideration together with the complex pattern of reflex effects from certain regions of the [illegible] skin Hagbarth 1952 and from the pad Engberg 1964 it is evidently difficult to exclude α [illegible] linkage even when the reflex effects do not follow the original description of Sherrington 1910 In order to elucidate this problem in more detail it is necessary to record in parallel effects to α and γ motoneurones in the same preparation.

In the present material reflex effects could be evoked in γ motoneurones whether they received descending monosynaptic effects from the brain stem or not Since there is no trace of excitation from the FRQ in the flexor γ motoneurone of Fig 6 this cell is probably of the dynamic type Bergmans and Grillner 1968a *cf* Discussion page 15 It is indeed powerfully excited from ipsilateral skin [illegible] and high threshold muscle afferents Correspondingly in the extensor [illegible] motoneurones in Fig 4 7 and 8 inhibition is evoked from these afferents as would be expected from the flexor reflex pattern. The excitation from cutaneous nerves of the G S [illegible] motoneurone in Fig 9 may be related to Hagbarth's finding that G S α motoneurones can receive excitation with short latency from the sural nerve also without preceding inhibition. It should be noted that the reflex effects from the knee joint nerve conforms to the reflex pattern evoked from the other peripheral nerves Fig 6 H 7 D 9 F)

After the functional subdivision of γ motoneurones into a static and dynamic type

(Matthews 1962), Alnaes *et al* (1965) with an indirect method demonstrated that in the spinal state dynamic but not static γ motoneurones can be reflexly activated on repetitive stimulation of peripheral nerves Bergmans and Grillner (1967, 1968b c) confirmed that dynamic flexor γ-motoneurones usually are more effectively activated from the FRA than the static ones but found some reflex activation also of static γ motoneurones However, on repetitive stimulation at 40/sec, which was used by Alnaes *et al* (1965), static γ motoneurones were not activated Hence it cannot be expected that single volleys in the FRA should excite exclusively dynamic flexor γ motoneurones

The present small and heterogeneous material does not allow any conclusion beyond the suggestion that dynamic γ motoneurones to flexors receive strong effects from the FRA and that these effects rather conform to the flexor reflex pattern It is therefore tentatively suggested that in the flexor reflex there is a linkage between α and dynamic γ motoneurones Whether a similar linkage also exists with static γ motoneurones cannot be determined until more information has been obtained on the reflex effects from the FRA to static γ motoneurones and in particular on the process responsible for inhibition of reflex transmission to them on repetitive stimulation of the FRA

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An Electromyographic Analysis of Muscular Activity in the Hindlimb of the Cat during Unrestrained Locomotion

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Abstract

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During walking in unrestrained cats the electromyographic activity in many hindlimb muscles been correlated with the angular movement in the hip knee and ankle joints The activity ier uniform in the extensors but individual in different functional groups of flexor muscles vations on the precise timing of the onset of the main extensor activity suggest that it is d reflex effect produced by stimulation of receptors (from muscle or skin) in the limb is assumed that the basic activity is a centrally programmed alternating activation of ex nsors and flexors *A possible reflex regulation mainly from Ia afferents of this basic activity* is discussed with special attention given to factors that may elucidate the difference in movement at the hip on one side and the knee and ankle on the other

Particularly in earlier investigations dealing with the reflex activity in the cat's spinal cord the results were often discussed in relation to the locomotion of the animal Thus Sherrington (1910) describes the flexion reflex not only as a protective withdrawal reflex but also when it is elicited from deep structures of the limb as a reflex of importance for the flexion phase of the alternating reflex act of stepping Further, when mentioning the extensor thrust reflex he points out that this "appears in several ways suited to play a part in the reflex step *Philippson* (1905) analyzed cinematographic films made by Marey of the dog's locomotion and he thoroughly discussed the possible reflex mechanisms underlying the different phases of the step However the temporal sequence of muscle activation in the natural movements of the animals have not been known and it seems important to have this information as a basis for the interpretation of certain reflex functions

Our interest was prompted by the finding that the Ia interconnexions between hip and knee muscles are more complex than those between knee and ankle muscles (R. M Eccles and Lundberg 1958 This was correlated with the fact that during stepping movements in the hip and knee joints do not occur in phase as they do in

knee and ankle joints and it was suggested that the more complex interconnexions had evolved to provide reflex actions that may assist locomotion This comparison of Ia linkage with limb movements was unsatisfactory because of the difficulty in assessing contributions from active to passive movements The present analysis of the muscular activity was started in order to obtain a firmer basis for further discussion Some of the findings have been briefly reported (Engberg and Lundberg 1962 Engberg 1964)

Methods

The experiments were performed on nine cats, selected to be co-operative The animals were enticed to walk or run about 4 m along a pathway and their movements recorded by a came [illegible]

[illegible] lled copper wires the insulation being scraped off about 1 mm from the end of the wire, which was bent to a hook During a short halothane narcosis these electrodes were inserted inside a hypodermic needle, which was then withdrawn, leaving the hook in place The localization of the electrode was controlled before and [illegible]

[illegible] 16 mm camera In a few experiments a 35 mm camera was used with continuously moving film The cat was then illuminated by a stroboscopic light giving 100 or 200 flashes per sec of high intensity and 3 μsec duration The time relations between the records of movement and EMG were accurately defined by a flashing neon bulb behind the moving cat synchronized with a time marker in the EMG recording system

Results

1 *Hindlimb movements*

In different speeds of locomotion the cat like other animals uses several different types of gait Walk trot and gallop are easy to distinguish and used for slow medium and fast locomotion respectively The present studies of muscle activities were done mainly in these three types of gait but intermediary types like amble rack or canter (*cf* Roberts 1967) were sometimes present and then included in the material as trot or gallop respectively To provide a basis for relating muscle activity to angular movements in different joints a series of films were taken with stroboscopic illumination (and thus fast exposure) of three different cats running at various speeds The instantaneous joint angles were then measured and plotted against time Fig 1 shows one example where the same cat exhibited gallop (A) trot (B) and medium fast walk (C) It is evident that despite the great differences between these types of gait the angular movements in the hip knee and ankle joints vary but little As the speed

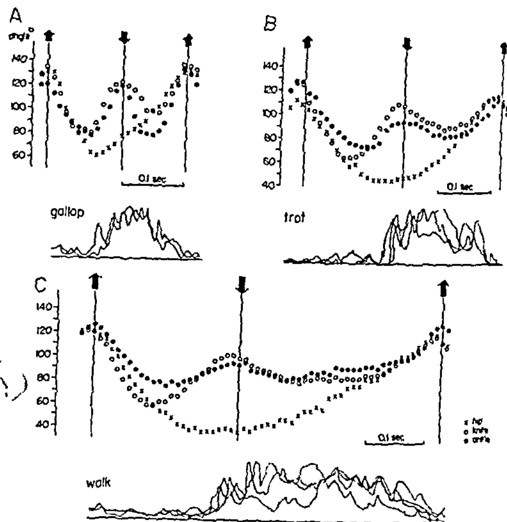


Fig 1 Angular movements in hip knee and ankle joints during gallop (A) trot (B) and walk (C) Below each graph are sets of superimposed integrated EMGs from gastrocnemius recorded at the corresponding types of gait Arrows above graphs indicate lifting (^) and placing (v) of the foot as in the following figures

of movement increases so does the over all excursion in any one joint but the sequential relations to the phases of the step cycle remain largely unchanged Philippson (1905) divided the step cycle in the dog into one flexion phase and three extension phases and it is feasible to use his terminology also for the cat the movements are very similar The flexion phase starts with the lifting of the foot (indicated by arrow ↑ on top of Fig 1 when flexion begins in all three joints and ends when extension begins in the knee and ankle This extension occurs when the leg is still in the air (swing phase and was termed the first extension phase The speed of hip flexion gradually decreases during this phase but except in the gallop

the hip does not extend until after the foot is placed on the ground (↓) Even in the gallop there is a clear phase lag between the leg foot extension and that of the hip The second and third extension phases make up the stance phase (when the foot is placed on the ground) Throughout this time the hip is extending with increasing speed in all three types of gait During the first part however the knee and ankle yield under the weight of the body and cannot maintain their extension particularly in the gallop Only in the third extension phase is there a similar increase in all three joint angles The outcome of these relative movements of the joints is that the hind part of the cat is kept approximately level during the whole stance phase

2 *EMG recording*

Extensors Below each graph in Fig 1 are illustrations of the activity in the gastrocnemius muscle recorded with corresponding time scales and types of gait The records consist of superimposed EMGs from several step cycles as described in the Methods section The main activity of this ankle extensor occurs during the three extension phases and it is particularly strong during the second extension phase There is no important difference between the gastrocnemius EMGs from gallop trot and walk except that the faster the animal moves the larger are the recorded potentials (Differences in amplitudes between sets of records are not shown in the illustrations see Methods) It is true that the EMG activity is shorter in relation to the step cycle in the gallop than in the slower rates as there is a decay starting already in the middle of the stance phase This is however largely due to shorter duration of the cycles the decay occurs generally in extensors during the last 50—100 msec of the stance phase (*cf* Fig 2) and this time may be correlated with the relatively slower decrease in muscular contraction force and with some deceleration of joint movement before the flexion phase starts

Fig 2 gives a comparison between the activities of gastrocnemius and three other extensors the knee extensor vastus lateralis and the two hip extensors adductor femoris and semimembranosus They were all recorded in trotting animals and they correspond approximately to the angle diagram on top The semimembranosus records were taken from that part of the muscle which inserts on the femur and which has no knee flexor action—there is a functional difference between the two parts of this muscle (*cf* below) All these extensors conform to the same pattern They have a definite burst of activity during the first extension phase more or less isolated from their main period of activity in the second and third extension phases The early period of activity deserves some particular attention Despite the difference in joint movements in this phase hip extensors as well as knee and ankle extensors display this initial activity However it starts later and is less marked in gastrocnemius and it is small or not present at all in the toe extensors flexor digitorum longus and brevis (Engberg 1964) When comparing the joint movement with muscular activity one must also consider the dynamic aspects of locomotion during the flexion phase the limb has been accelerated to a forward swing movement that must be decelerated before the foot is placed This deceleration is

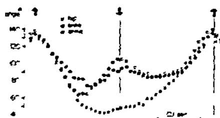










Fig. 2. EMGs from four hindlimb extensors related to the angular movements in hip, knee and ankle joints. The records are from different experiments, but all from cats trotting at approximately the same speed. Marks under the middle of each record indicate the moment of placing of the foot.

brought about in the first extension phase by activity in the hip extensors. The concomitant activity in knee extensors has no dynamic force to counteract—indeed this force adds to their contractile force and produces a knee extension that increases the length of the stride. This movement is probably counterbalanced and eventually decelerated by the knee flexors (see below). The cat being digitigrade then uses the ankle extensors to oppose the dynamic forces resulting from the deceleration of the forward swing of the limb just before the foot is placed and thus lowers the foot slightly to place the pads on the ground.

The main extensor activity in the second extension phase often has a rapid onset in connexion with the placing of the foot (the placing is indicated by a vertical bar under the middle of each EMG). The exact time relations of this onset were studied with a higher time resolution. Fig. 3 illustrates the results obtained with two different methods. In A the direct and integrated EMG from vastus lateralis can be related to five successive frames of a stroboscopic film (see Methods for details). The cat runs on a polished aluminium surface which gives a mirror image of the right hindfoot studied. There is no doubt that the foot is still in the air in frame 2 and it barely touches ground in frame 3 when the EMG activity has already attained

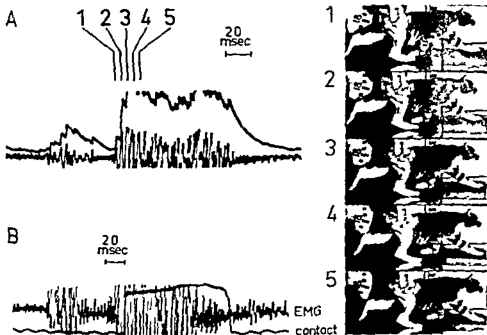


Fig 3 Time relations between foot contact with ground and extensor EMG Records in A and B from vastus lateralis A Upper beam integrated EMG lower beam direct record To the right is a short sequence of pictures taken at 200 sec simultaneously with the EMG record Figures on top of record indicate the moment of exposure for each of the pictures The cat was running on a polished aluminium plate B Without the integrated EMG the spare beam was used to indicate foot contact with ground (see text) upwards deflexion when the foot is put down

its maximum The muscle activity starts at the time of frame 1 when all the four feet are in the air

In the experiment of Fig 3 B the cat was connected electrically to a wire suspended in a spring from the ceiling The pads of the right hindfoot had been gently rubbed with sandpaper and smeared with electrode paste whereas the other feet were coated by sheaths of very thin rubber With the cat running on a metal surface placing of the foot closed an electric circuit shifting the lower beam of the oscilloscope upwards On the upper beam the direct EMG of vastus lateralis of the same leg was recorded the results confirm those of Fig 3 A The method was used also with recording from adductor femoris and gastrocnemius and during different speeds of locomotion In trot and gallop the EMG activity of all three extensors usually preceded the placing of the foot to about the same extent as shown in Fig 3 Only when the cat walked slowly was the onset sometimes simultaneous with or slightly later than the placing

Flexors The typical activity of knee and ankle flexors is illustrated by the records from semitendinosus in Fig 4 Other flexors investigated biceps posterior gracilis tibialis anterior peroneus longus and extensor digitorum longus gave rather ...

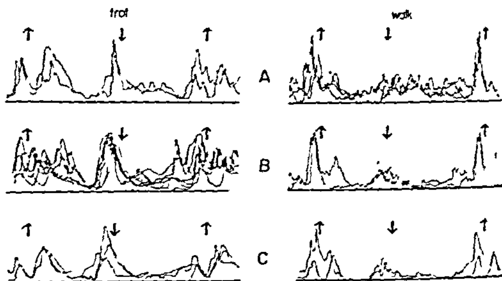


Fig 4 EMGs from semitendinosus at different speeds of locomotion. Three different experiments A from one cat B and C from another Records to the left rather fast trot right walk Different time scales are used for the left and right records giving the same step cycle length.

records (cf Engberg 1964 Fig 22) The regularity of the EMG patterns is shown by records from three different experiments (A B and C) In walking (right hand records the knee flexor is strongly activated for a short period at the end of the third extension phase This burst of activity is found in all flexors investigated except extensor digitorum brevis (Engberg 1964) There is also some activity in the beginning of the flexion phase this activity gradually decreases and except for a slight increase when the foot is being placed there is little activity for the rest of the cycle In trotting the activity is generally much stronger and in the left row of records the amplitudes had to be reduced As in walking there is an onset of activity at the end of the third extension phase but this is followed by another strong activation in the flexion phase and the muscle is only relaxing for a short period (cf relation of step cycles to time scale in Fig 1 before a very strong burst of activity at the end of the first extension phase The very striking increase in flexor activity in the first extension phase may be needed for a deceleration of the more rapid forward swing of the leg in the trot A burst of activity in this phase is found in flexors at all joints except at the hip Fig 5 A shows that the hip flexor iliopsoas is inactive in this period which would be expected since contraction of this muscle would accelerate the forward swing The EMG of iliopsoas is different also in the flexion phase the activity starts somewhat later and is more prolonged particularly in walking when it is maintained until the foot is placed (Fig 5 B) This parallels the prolonged flexion of the hip as compared to that of the peripheral joints

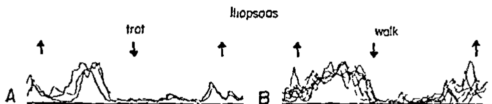


Fig 5 EMGs from iliopsoas in trot (A) and walk (B)

Bifunctional muscles Several muscles were investigated, which have anatomically opposite functions at two joints the lateral part of sartorius inserting on the patella (hip flexor, knee extensor), rectus femoris (hip flexor, knee extensor), the part of semimembranosus inserting on the tibia (hip extensor knee flexor), semitendinosus (hip extensor, knee flexor) and gastrocnemius (ankle extensor, knee flexor) Of these muscles, the latter two behave as expected from their main functions and reflex connexions, *i e* as a flexor and an extensor respectively, but lat sartorius, rectus and semimembranosus could not be simply classified In the walking cat the EMG from the lateral sartorius (Fig 6) is rather like that of a flexor with activity in the last part of the third extension phase and, like iliopsoas in the flexion phase In trot the activity in the flexion phase and sometimes (lower left record) in the third extension phase, also follows the flexion pattern, but there is an additional extensor type activity in the second extension phase The tibial part of semimembranosus (Fig 7) has a somewhat similar double pattern The speed of motion decreases from

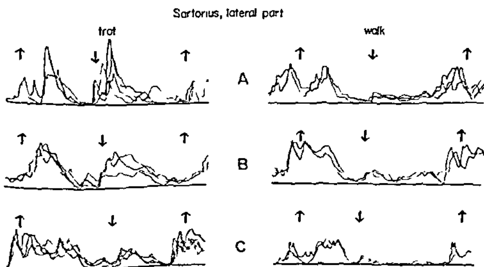


Fig 6 EMGs from lateral part of sartorius at different speeds of locomotion As in Fig 4 but for three different cats Note the increased activity in the extension phases that appears in the trot

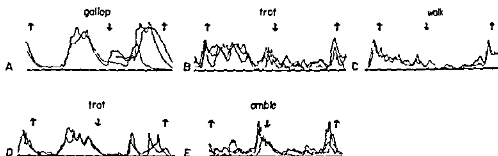


Fig 7 EMGs from semimembranosus (tibial part) at different speeds of locomotion A D and E from the same experiment on one cat B and C from another cat A gallop B and D trot C walk E amble Note the similar patterns in A and D that change to that in E for the slow ambling

left to right in the figure In trot, amble and walk (B E and C) the muscle usually behaves like semitendinosus but in gallop (A) and sometimes in trot (D) there is a change towards more activity in the late extensor phases and the activity during the swing phase is delayed so that it occurs mainly in the first extension phase

Rectus also displays an irregular EMG pattern In Fig 8 A and B from the same experiment the direct EMGs from the distal part of the muscle are shown with foot placing indicated as in Fig 3 B The cat was at first walking (A) but in the next run trotting (B) In both cases there is a shortlasting activity early in the first extension phase much as is typical of the knee extensors In A there is then a long interval and the muscle is only activated again in the third extension phase relaxing with the extensors some 50 msec before the foot is lifted There is no sign of the very late stance phase activity typical of the flexors In B the main activity starts earlier in the stance phase and the EMG is more like that of vastus lateralis Records from the proximal part of rectus (C D also from the same experiment) display typical extensor type EMGs during the stance phase in trot and gallop but also activity in the flexion phase Further the activity of the first extension phase seen in A seems to have moved in C to an earlier position in the cycle Records E and F from other experiments show essentially the same atypical extensor activity as B and A respectively but there are also particularly in F signs of the flexion phase activity It is not known if in these double joint muscles a different pattern of activity can be found in the same motor units with a change of the gait or whether the flexor like activity is evoked in one group and the extensor like activity in another group of motor units

Discussion

The present results show that the general classification of hindlimb muscles in the cat as flexors or extensors based on their participation in the flexion reflex crossed extension reflex and in spinal stepping (Sherrington 1910) is applicable for many

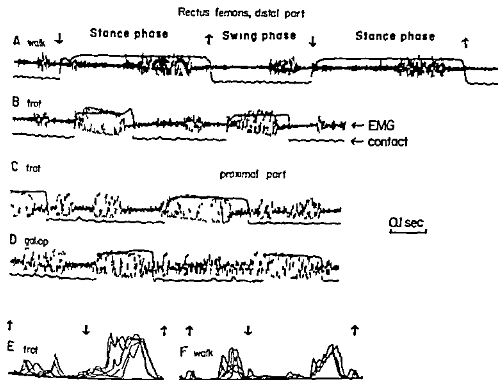


Fig 8 EMGs from rectus femoris at different speeds of locomotion A—D from the same experiment (recorded without integration and with foot contact indication as Fig 3 B) A distal part of rectus walk B distal part of rectus trot C proximal part trot D proximal part gallop E and F superimposed integrated records from proximal rectus in trot and walk respectively Note the increasing extensor type activity when the cat moves faster

muscles also when their natural activity in the step is studied The activities of individual muscles in the flexor group do not however conform to a single pattern like those of extensors The extensors always have their main activity in the stance phase in most cases preceded by a shortlasting activity in the first extension phase The knee and ankle flexors behave rather uniformly They are always strongly activated for a short period at the end of the stance phase followed by some activity in the early flexion phase and another short burst late in the first extension phase Two flexor muscles must be classified separately iliopsoas and extensor digitorum brevis The hip flexor iliopsoas is more active in the flexion phase than the other flexors and it does not have their strong isolated burst of activity just before the foot is placed Extensor digitorum brevis on the other hand is maximally active during the placing of the foot and differs from all other flexors in being relaxed in the flexion phase and the third extension phase (Engberg 1964) Some of the double joint muscles have a more complex activity pattern and cannot be classified as pure flexors or extensors The activities of different types of muscles in

relation to the limb movements they may give rise to have been discussed under Results.

In the following discussion of a possible reflex control of stepping brief reference will be made to effects from cutaneous receptors but the main part will be devoted to effects from large muscle spindle afferents and Golgi tendon organ afferents. The patterns considered will be those found in the low spinal animal (Eccles, Eccles and Lundberg 1957 a, b, R. M. Eccles and Lundberg 1958, 1959). This may be an oversimplification, particularly with the effects from Ib afferents since more complex patterns are found in the high spinal cat (Laporte and Lloyd 1952) or under the descending facilitatory influence (Hongo, Jankowska and Lundberg 1968). Another basis for the discussion will be some assumptions regarding the γ system. There have been indications that the γ-activity is of considerable importance in stepping (Thulin 1960). The elegant experiments by von Euler and his collaborators reveal that during respiration spindle afferents from the intercostal muscle discharge maximally when the muscle contracts and not in the respiratory phase when the muscle is stretched (Critchlow and von Euler 1963, von Euler 1966). This shows that there is a very effective alternating fusimotor activity during respiration. Recently published very interesting findings suggest that a similar situation exists in the control of [illegible]. The experiments have been made on mesencephalic cats in which seemingly normal stepping is produced by stimulation of a certain region in the midbrain if the limbs are set in motion by a treadmill (Shik, Orlovskii and Severin 1966, Orlovskii, Severin and Shik 1966, Severin and Orlovskii 1967). Records from muscle spindle afferents (Severin, Orlovskii and Shik 1967) have revealed that in extensors the highest frequency is found during the stance phase, in particular during the yield in the second extension phase. Spindle afferents from flexor muscles discharge maximally during the flexion phase but the frequency is not as high as in afferents from extensors and there is also some discharge during the stance phase when the muscle is passively stretched. These experiments show that during muscle contraction in stepping there is an effective fusimotor activity. It may be argued that stepping on a treadmill in mesencephalic cats may not be representative for stepping in the intact animal, but while this may hold true for details in the reflex control it is unlikely that the basic mechanism may be very different. The findings by Severin *et al.* (1967) are obviously of paramount importance in evaluating the contribution the reflexes from Ia afferents may have in the control of stepping. As a basis for the following discussion of reflex contribution from Ia afferents during stepping in the intact cat it will be assumed that the highest discharge frequencies are reached during contraction of the muscles and not in the phase when they are passively stretched by contraction of their antagonist.

Sherrington (1910) investigated the rôle of reflexes in the foot [illegible] proprioceptive or elicited from cutaneous receptors by denervating the foot. He found virtually no change in the natural standing or walking of the animal except for changes in the position of the toes that may be explained by the motor denervation of toe muscles. The effect of receptors in the central pad has been excluded by infiltration of the

pad with lidocaine in one of our experiments on vastus lateralis and in some experiments on the foot muscles (Engberg 1964)—in no case was there any significant alteration in the pattern of muscular activity From experiments in man Hoffmann (1922) concluded that the stretch reflex contributed to ankle extensor activation and his EMG records show an increased activity some 50 msec after the foot placing Our finding that the main activity of extensors in the cat's hindlimb starts shortly before the foot touches the ground eliminates reflexes due to pressure on the planta and Ia reflexes caused by stretch of the extensors when the foot is placed as a cause of the onset of the main extensor activation.

It is likely that Ia reflexes from the extensor muscles may play a role in maintaining the activity during the stance phase In particular, it would be expected that the yield in the knee, ankle and toe extensors in the second extension phase would give an intense Ia discharge from these muscles (Severin *et al* 1967) The comparison of activity in the hip extensors, adductor femoris and semimembranosus was of particular interest because of the Ia receptiveness of their motoneurones (R M Eccles and Lundberg 1958) They are all Ia excited from hip extensors but in addition adductor femoris motoneurones receive Ia excitation from knee extensors and semimembranosus motoneurones from knee flexors However, the finding that there is no obvious difference in the EMG of these muscles during the stance phase does certainly not exclude that Ia reflexes may play a role in their activation

Our study of the precise timing of the onset of extensor activity during placing of the foot has led us to view that the extensor activity essentially is centrally programmed The neuronal system described by Jankowska *et al* (1967 a, b) is of particular interest in this connexion This system appears to be organized by means of a double reciprocal innervation at an interneuronal level, for central generation of rhythmic alternating activity in alpha and gamma motoneurones to flexors and extensors much as postulated by Graham Brown (1911) in his hypothesis of the paired half centres in the spinal cord If extensor activation essentially is part of a centrally programmed process a corresponding activity might be expected in the flexors in the other phase of the step The simplest model would give synchronous activation of all extensors in the stance phase and synchronous activation of flexors in the swing phase To a large extent this simple model can be used to explain the activity in extensors since during the stance phase there is an essentially identical activity in all the extensors investigated In flexor muscles on the other hand the activity does not follow a single pattern but is individual in different functional groups of flexors Only in the hip flexors the activity conforms reasonably well to that expected from the simplest model for alternating rhythmic activity—during the swing phase of the step they may display a sustained activity reciprocal to the extensor activity during the stance phase By comparison the activity in the knee flexor semitendinosus is poorly sustained in the swing phase From an initial maximum it gradually declines during the flexion phase

This difference between iliopsoas and semitendinosus we will now consider in view of the Ia interconnexions between hip and knee muscles The pure hip flexor

iliopsoas receives heteronymous Ia excitation from rectus, sartorius and tensor fasciae latae which all flex the hip (R. M. Eccles and Lundberg 1958). Rectus and portions of the two latter muscles also extend the knee. We have not recorded the EMG from tensor fasciae latae but sartorius (both lateral and medial) and to some extent also rectus are activated during the flexion phase. On the basis of the assumption that they receive an increased fusimotor activity in this phase it is postulated that the stretch produced by the knee flexion will give a high frequency Ia discharge. Hence these double joint muscles would resemble the extensors (during the second phase of extension) in that they are stretched against an increased fusimotor activity. The Ia discharge would add to the basic central excitation and produce a more sustained activation both of the pure hip flexors and the double joint muscles which flex the hip and extend the knee. In this comparison of iliopsoas and semitendinosus we also have to consider the Ia inhibitory effects. Ia volleys from sartorius, rectus and tensor fasciae latae produce large disynaptic IPSPs in semitendinosus motoneurones (R. M. Eccles and Lundberg 1958). Hence both with regard to the excitatory and inhibitory action it is likely that the Ia interposition of these double joint muscles between the pure hip flexors and the knee flexors may contribute to a different time course of the activity in iliopsoas and semitendinosus and thereby explain how the limb can be moved forward by flexion at the hip during most of the swing phase while flexion at the knee (and ankle) occurs only in the first half of this phase. The possible function of double joint muscles in initiating the first extension phase will be discussed below.

Proprioceptive reflexes may also in other respects be of importance in stepping. Towards the end of the stance phase activity in spindle afferents from flexors might give excitation of flexor and reciprocal inhibition of extensor motor nuclei. One would also expect activity in Golgi tendon organ afferents from extensors in the stance phase (*cf.* Severin *et al.* 1967) contributing to the cessation of extensor and onset of flexor activity. It is noteworthy that of the flexor muscles tested extensor digitorum brevis alone lacks activity in this phase (Engberg 1964). Engberg has pointed out that shortly before the foot leaves the ground the leg is maximally stretched in all its joints except the toes, which are dorsiflexed under the weight of the body. Hence a Ia stretch reflex would not be expected in extensor digitorum brevis during this phase of the step. Another characteristic feature in many flexor muscles is *the short burst of activity late in the first extension phase*. Presumably this burst follows the extensor activity which produces the first extension phase in the latter half of the swing phase. This short burst is possibly also evoked from extensor tendon organ afferents and flexor spindle afferents. It should be noted that the hip is not extended in this phase and that the hip flexors are not excited (as can be seen from the records obtained during trot in A, Fig. 5.

Special aspects of the reciprocal Ia inhibition has been discussed above but it remains to consider its more general role. In mesencephalic cats the discharges in spindle afferents from flexors do not seem to reach as high frequencies during the swing phase as those found in afferents from extensors during the second extension

phase (Severin *et al* 1967) It is therefore possible that reciprocal Ia inhibition of flexor nuclei during the stance phase is more important than the corresponding effect to extensor nuclei during the swing phase The reciprocal Ia inhibition of flexor nuclei may play an important role during the stance phase because even if the flexors do not receive programmed activation during this phase they could be reflexly activated for example from Ib afferents of extensors In this connexion it should also be noted that in mesencephalic cats there is some activity in Ia afferents from flexors during the entire stance phase (Severin *et al* 1967) The reciprocal Ia inhibition of flexors is assumed to be particularly important during the second phase of extension because of the high frequency of the Ia discharge produced by yield under the weight of the body It is of considerable interest that a corresponding yield is not found in the hip extensors (*cf* Fig 1 and Results) Hip flexor motoneurones may therefore in this phase receive less Ia inhibition from their strict antagonists than the motor nuclei of flexors at the knee and ankle Nevertheless the hip flexor motor nuclei may be effectively Ia inhibited since these nuclei are exceptional in that they receive Ia inhibition *not only from the hip extensors but also from the knee extensors* (R M Eccles and Lundberg 1958) Since previously no suggestion has been forwarded regarding the functional significance that this Ia inhibitory connexion to hip flexor nuclei may have it is of some interest that it can be correlated with the special mechanical situation at the hip as compared with the other joints, where Ia inhibition is organized along the more simple one joint pattern Extensor digitorum brevis is exceptional also during the first half of the stance phase in that, particularly during fast running, this muscle does not relax when the foot is put down—the activity may last almost as long as in the extensors (Engberg 1964) For this reason it would be of interest to know if extensor digitorum brevis motoneurones do receive reciprocal Ia inhibition from antagonist muscles—there does not seem to be any information in the literature regarding this

The reciprocal Ia inhibition from flexors during the swing phase is of interest when considering the first phase of extension which is an essential but with respect to its origin very puzzling feature in the gait of the cat In slow walking this phase is absent in the hip but it is always pronounced in the knee and ankle joints The extension has an earlier onset at the knee than at the ankle and when it occurs in the hip this is even later During the first extension phase there is activity both in hip ankle and knee extensors This activity may be a Ia reflex caused by the stretch of the extensors during flexion of limbs only if the reciprocal Ia inhibition from the flexors is weak or absent The activity in vasto-crureus does not seem to be early enough to account for the onset of knee extension but the obvious limitations in our technique does not allow a definite conclusion The contraction of the double joint muscles lat sartorius and rectus could be instrumental in starting the first extension at the knee joint (*cf* Fig 8) but if so a reflex origin of the activity in vasto-crureus is difficult to imagine Whatever role the double joint muscles and vasto-crureus play for the onset the knee extension would stretch the double joint portion of gastrocnemius and may produce ankle extension by a stretch reflex in this muscl

(observe that the linkage in the Ia inhibitory pathway from ankle flexor to extensor is very weak, R. M. Eccles and Lundberg 1959). Such a sequence would explain the time lag between the knee and ankle extension. Further experiments are required to decide the role of double joint versus single joint muscles in producing the first extension.

It is also necessary to consider the importance that crossed reflexes (and reflexes from the forelimb) may have for stepping. If they were of major importance one would expect the pattern of muscle activity to vary from one type of gait to another. In the pure flexors and extensors this is not the case. Even when the cat shifts from trot to gallop and the relation between the movements of different limbs changes completely, the main feature of the EMG remains unaltered. This does, of course, not exclude that the afferent input may be important for the onset and maintenance of the cyclic activity (Sherrington 1910, Shik *et al.* 1966).

Before closing this discourse on stepping we would like again to emphasize its entirely speculative character. It must also be clearly stated that our scheme does not take into account a number of known spinal reflex actions: for example neither reflexes from spindle afferents with secondary endings nor any effects to primary afferent terminals have been discussed. We have neither considered the possibility that the centres responsible for the basic rhythmic activity may be influenced by receptor activity (*cf.* Jankowska *et al.* 1967 a, b) nor the supraspinal control of reflex pathways or interactive mechanisms between reflex pathways (Lundberg 1966). Special references should, however, be made to recurrent inhibition from motor axon collaterals. Severin, Orlovskii and Shik (1968) have observed a depression of transmission in the recurrent inhibitory pathway to motoneurones during stepping in mesencephalic cats. A corresponding inhibition is found (Bergmans, Burke, Fedina and Lundberg, to be published) during activation of the neuronal pathway which may give alternating activation of extensors and flexors (Jankowska *et al.* 1967 a, b). It has recently been shown that the Ia inhibitory pathway to motoneurones is inhibited from motor axon collaterals (Hultborn, Jankowska and Lindström 1968 a, b). For operation of Ia inhibition in locomotion as outlined above in this discussion it may therefore be of paramount importance that recurrent inhibition is blocked during stepping.

To sum up, stepping may require a centrally programmed alternating activation of flexor and extensor possibly from centres organized as those described by Jankowska *et al.* (1967 a, b). Such an activity can well explain the main activity of extensors while their activity in the first extension phase is more difficult to understand; possibly it may be caused by proprioceptive reflexes. It remains to be seen whether the more differentiated pattern in the various functional groups of flexor muscles can be explained—as has been attempted above—by assuming a common centrally programmed activation on which proprioceptive reflexes are superimposed, or whether the central program for flexor activation differentiates between various functional groups of muscles. Special attention has been given to the role of double joint muscles which flex the hip and extend the knee. We have tried to ma...

how proprioceptive reflex actions could provide for a differential activation that is required to produce the normal gait. Furthermore such reflexes could provide rapidly the adequate adjustments that may be required for example from changes in the ground configuration. The stretch reflex may serve as an example of such a function being interposed as a link in a servo system (*cf* Merton 1953, Granit 1955, Matthews 1964). The interneuronal centres described by Jankowska *et al* (1967 a, b) are known to influence strongly the fusimotor system (Grillner, Hongo and Lundberg 1967, Bergmans and Grillner 1967) and we have discussed above how the rhythmic movements to some extent could be produced by use of the gamma loop as has been shown for the activation of respiratory muscles (von Euler 1966) and during stepping in mesencephalic cats (Severin *et al* 1967). This would provide means of automatic compensation for the various forces counteracting the progression of the animal.

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Acta physiol. scand. 1969. 75. 631—635

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Brain Monoamines in the Temperature Acclimation of Mice

By

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Abstract

HARRI, M. and R. TIRRI. *Brain monoamines in the temperature acclimation of mice.* Acta physiol. scand. 1969. 75. 631—635.

The brain 5-hydroxytryptamine (5-HT) and noradrenaline (NA) levels were determined fluorimetrically from mice during prolonged exposure to cold or heat. The NA level did not change until at extreme ambient temperatures, in which cases, especially in heat, a marked decrease was observed. In cold environment the 5-HT level decreased after an initial short increase. However, within one day the initial control level was reached again. In warm environment the changes were reversed. Some depletion experiments, where an inhibitor of 5-HT synthesis was used, showed that the activity of brain 5-HT neurons decreased for some days in cold, but returned gradually to the control level after the animals had acclimated to cold.

Prolonged exposure of homeothermic animals to cold environment is known to induce various adaptive responses, e.g. an increase in non-shivering thermogenesis as well as in noradrenaline sensitivity and excretion (Depocas 1960, Héroux 1963 and Leduc 1961). However, little is known about the central nervous mechanisms of cold acclimation (Slonim 1963). The role of the hypothalamus in the thermoregulation involved in the maintenance of homeothermy is well established. The noradrenaline (NA) and 5-hydroxytryptamine (5-HT, serotonin) release in the brain especially in the hypothalamic neurons was shown to be strongly affected by short exposures to cold or heat (Corrodi, Fuxe and Hokfelt 1967).

Thus these transmitters probably have an important role as mediators of central thermoregulatory processes as suggested by Feldberg and Myers (1963, 1964). The activity of brain NA neurons was found to increase during cold stress (Goldstein and Nakajima 1966) or heat stress (Corrodi, Fuxe and Hokfelt 1967). Heat treatment enhanced the activity of 5-HT neurons while cold had the opposite effect. In these studies the depletion of these substances after inhibition of their synthesis was used as a criterion of the activity. So far no attention has been paid to the possible effect of the temperature induced alterations of the brain monoamine metabolism in the initiation of the peripheral adaptive changes associated with cold acclimation. The present experiments were carried out in order to elucidate the time-course on the brain monoamine metabolism in temperature acclimation.

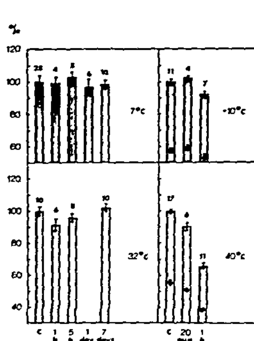


Fig 1

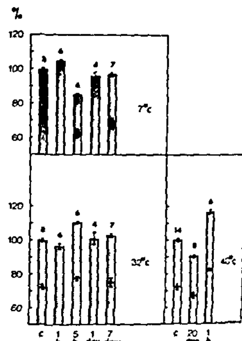


Fig 2

Fig. 1 The level of NA in mouse brain after exposure of animals to various ambient temperatures. The data are expressed as percentage of control values ± SE (C = 0.41 µg/g at 7° C, 0.40 µg/g at −10° C, 0.42 µg/g at 32° C, 0.40 µg/g at 40° C). The number of experiments are given on the top of each column. The duration of the exposure is indicated at the base of the columns. The upper part of divided columns represents the "free" form and the lower part the "bound" form of NA. C = control animals kept at 22° C.

Fig. 2 The level of 5-HT in mouse brain after exposure of animals to various ambient temperatures. (C = 0.67 µg/g at 7 and 32° C, 0.66 µg/g at 40° C.) Other explanations as in Fig. 1.

Material and methods

Altogether 290 NMRI male mice aged 3–5 months were used in the experiments. The ambient temperature and the duration of treatment is indicated in each figure. The animals were killed by decapitation and the brains were homogenized in ice-cold 0.1 M hydrochloric acid or 0.4 M perchloric acid and frozen. Assays of noradrenaline from perchloric acid homogenates were made with the procedure of Anton and Sayre (1962) using the fluorometric determination technique of Häggendal (1963). Serotonin assays from hydrochloric acid homogenates were performed by the method of Bogdanski *et al.* (1956) as modified by Kuntzman *et al.* (1961). When "free" and "bound" amines were separately determined the homogenization was made in 0.32 M sucrose. The determinations of "free" amine from the supernatant and "bound" from the crude mitochondrial fraction were carried out after a centrifugation for 30 min at 11 000 g. The α-propyl-dopacetamide (H 22/54) was used as an inhibitor of 5-HT biosynthesis (Carlsson, Corrodi and Waldeck 1963). The dose of 500 mg/kg dissolved in 50 % ethanol was administered to the animals intraperitoneally three hours before killing. Control determinations were always performed together with the assays from the experimental animal.

Results

Fig. 1 shows that the brain NA level did not change significantly until at extreme ambient temperatures. Even then the cold environment (−10° C) caused only a slight decrease (P<0.1), although moderate hypothermia (32—34° C) was induced

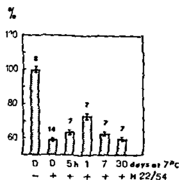


Fig 3

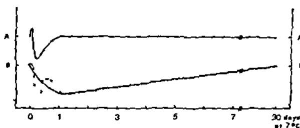


Fig 4

Fig 3 The 5 HT level in mouse brain after prolonged exposure of animals to 7° C when the 5 HT synthesis was inhibited by i p injection of H 22/54 (500 mg/kg) 3 hrs before killing The data are expressed as percentage of control values ± SE (=first column, 0 61 μg/g animals received an injection of the solvent only and were kept at 22° C for 3 hrs when kept at 7° C the values were about the same 0 65 ± 0 010) Second column represents the animals which received an injection of H 22/54 and were kept at 22° C The other columns show values from animals exposed to 7° C for periods of different durations and treated and killed at that temperature

Fig 4 Summary of the data of the brain 5 HT metabolism during cold acclimation in mice Upper curve 5 HT level at 7° C Lower solid curve 5 HT depletion rate at 7° C Broken curve Estimated 5 HT synthesis rate at 7° C A=5 HT level at 22° C B=5 HT depletion and synthesis levels at 22° C

by this temperature A much stronger change in the same direction however was observed when the animals were exposed to heat (40° C) in which case they were rendered hyperthermic (40—42° C) The decrease in 'free and bound NA was proportionally equal and the relationship between these forms was thus unchanged

The results from the brain 5 HT level are illustrated in Fig 2 The 5 HT content seems to be more dependent on ambient temperatures than the content of NA At 7° C an initial increase ($P<0\ 1$) appears within 1 hr This is followed by a pronounced decrease in the 5 HT level ($P<0\ 001$) at 5 hrs As soon as after one day the content returns to the initial control level A mirror image of this phenomenon was observed when the animals were transferred from room temperature into 32 or 40° C An initial decrease ($P<0\ 1$ at 32° C and $P<0\ 01$ at 40° C) was in both cases followed by an increase ($P<0\ 01$ and $P<0\ 001$) At 32° C this process occurred more slowly than at 40 C and the amine content returned to the control level in one day At 40° C the animals succumbed within 1 1/2 hrs In all groups the changes in the free and bound" forms of 5 HT occurred in the same proportion These results can be explained on the basis of depletion experiments as presented in Fig 3

The results in Fig 3 indicate that when the 5 HT *biosynthesis was inhibited* in mice at room temperature the brain 5 HT level dropped to about 60 per cent After exposure of animals to cold the depletion of the brain 5 HT level decreased which is shown by the increased content of this amine ($P<0\ 01$ cf 2nd and d column) This means that the activity of 5-HT neurons was gradually r

cold environment. At the 7th day, however, when the mice already are fairly well acclimated to cold (Lagerspetz 1963), the depletion of 5-HT was almost back to the initial level.

Discussion

The brain NA level did not change until at extreme ambient temperature. In these cases, especially in heat, a marked decrease was observed. It is obvious that these NA changes are due to stress rather than to specific temperature effects. Corrodi, Fuxe and Hokfelt (1967) were also lead to this conclusion by the results of their brain NA depletion experiments with rats kept at high temperatures. Although this non-specific stress reaction is perhaps not important for animals living in normal conditions, it might be of value at temporary extreme temperatures where in addition to the more specific temperature acclimation reaction also symptoms of general adaptation syndrom develop. Thus, the daily exposure of mice to severe cold (−20° C) for only 15 minutes improves the resistance to cold (Lagerspetz unpublished). Recently it has been shown by Ingenito (1968) that after prolonged cold exposure of rats (for 15 and 30 days) a slight increase in the brain NA was observed. Since the increase was small and seems not to be restricted to the hypothalamus, it was suggested that this increase occurs in sympathetic vasomotor nerves rather than at synaptic terminals of central neurons. The changes in free and bound forms of NA and 5-HT occurred in the same proportion and the relationship between them was thus unchanged.

A summary of the data concerning the brain 5-HT metabolism during cold acclimation is presented in Fig. 4. The upper curve, which shows the brain 5-HT level after exposure to cold, indicates that the short initial increase in the content is followed by a marked decrease. Within one day, however, the control level is reached again. The lower solid curve shows the decreased rate of 5-HT depletion during the first days after exposure to cold, which means that the activity of 5-HT neurons is reduced. The depletion rate returns only gradually back to the control level. The rate of the brain 5-HT synthesis, which is estimated on the bases of the 5-HT and the depletion rate, is shown by the broken curve. After exposure to cold the activity of the 5-HT neurons decreases soon, while the change in the rate of the 5-HT synthesis follows it after some delay. This causes a small increase in the 5-HT content at the beginning of cold exposure. After the control of the synthesis is readjusted a rapid lowering in the synthesis follows and later a downward "overshoot" occurs. This is the reason for the decrease in the 5-HT level. Later the synthesis and the utilization of 5-HT are balanced so that the content remains stable on the control level. Due to the changes observed in 5-HT levels in warm environment, the progress of events in heat acclimation may be proposed to occur likewise, but in the opposite direction. It can be concluded that the observed temporary changes of 5-HT metabolism which appear very soon after alteration in the ambient temperature may be the primary reactions which initiate the peripheral adaptive changes associated with acclimation to different ambient temperatures.

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Cerebrospinal Fluid Perfusion, A Method for the Study of Neurosecretory Organs

By

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Abstract

HIRVONEN, J., L. K. J. KARLSSON and Y. SALORINNE. *Cerebrospinal fluid perfusion, a method for the study of neurosecretory organs.* Acta physiol. scand. 1969. 75. 636—640.

The cerebrospinal perfusion method was applied for the study of the hypothalamo-neurohypophyseal secretory system and subcommissural organ in the cat and rat. The artificial cerebrospinal fluid was introduced into the lateral ventricle and taken out of the suboccipital cisterna. Hyperkalemic (8 meq/l) perfusion fluid effected a decrease of the aldehyde fuchsin positive material in the subcommissural organs of the cats and rats. Hyperosmotic perfusion fluid had such an effect neither on the neurohypophysis nor the subcommissural organ. The method seems usefull for the study of the cellular reactions of the neurosecretory organs *in vivo*.

The so-called neurosecretory areas or organs lie very close to the cerebrospinal fluid (CSF) system. The area around the hypothalamic structures, nucleus supraopticus (NSO) and nucleus paraventricularis (NPV), as well as the infundibular recess area, have been shown to be more permeable to the passage of various substances than most other areas of the brain (*cf.* Davson 1967).

The subcommissural organ, which histologically displays all the characteristics of a secretory gland, is literally bathed in cerebrospinal fluid. The study of the secretory phenomena in these organs has proceeded in many different ways but so far it seems that the possibility of using CSF perfusion has escaped the attention of investigators in this field although ventricular perfusion (introduced by Leusen 1948) is frequently used in physiological and pharmacological studies. In neurosecretory experiments the main advantage of CSF perfusion is that parts of the neurosecretory system can be affected more locally and specifically, while peripheral effects can be recorded independently. In the following some of the possible methods as applied to the cat and rat are described and preliminary results are given.

Material and methods

1. Perfusion of the cat CSF system

Altogether 25 cats of both sexes, weighing around 2.5 kg, were anesthetized with i.p. Nembutal (30 mg/kg b.w.). A polythene tube was then inserted into the lateral ventricle through a hole bored with a trephine in the parietal bone 6 mm laterally of the sagittal suture and 3 mm



Fig 1 A schematic sagittal section through the cat brain showing the position of the perfusion cannulas Inflow cannula in the lateral ventricle The cannula and the coupling needle are fixed to the parietal bone with small screws and dental cement The outflow needle pierces the atlanto-occipital membrane just below the occipital bone

behind the coronary suture (Feldberg and Sherwood 1953 Feldberg and Fleischhauer 1965) This technique has been criticized by Davson *et al* (1962), as erroneous injection into the subarachnoid space is too easily apt to occur but in the present experiment this presented no problems since the position of the tube could be verified accurately (see below) A coupling needle was inserted into the tube and the aggregate then fixed to the parietal bone with small screws and dental cement As the last operative procedure another needle was inserted into

ventricular system at a pressure of approximately 150 mm H_2O which still lies within physiological limits *according to many investigators (cf Davson 1967)*, and produced a flow of 0 5—1 ml/min This perfusion was continued for two hours The head of the cat was fixed with ear pins and a nose ring It was sometimes necessary to give an additional 0 5—1 mg % Nembutal to the animal

The cats were divided into 5 equal groups (Table 1) The cats of the two control groups (1 and 5) were perfused with artificial fluid (see below) containing 5 meq K/l (Group 1) and 0 9 % NaCl solution, (Group 5) respectively An osmotic stimulus was effected with 1 8 % NaCl solution (Group 2) and also with 2 6 % urea solution (Group 3) to give twice the normal osmotic pressure The effect of ionic changes was tested by varying the K con centration (Group 4)

The experiments were started with perfusion of the cat which is a fairly large animal The results obtained with the hyperkalemic solution were re tested with larger groups of rats smaller and less expensive animals

2 Perfusion of the rat CSF system

They received
it through
ventricle
e Fig 2)
This flow
was maintained for one hour The perfusion fluid was a solution made to resemble the cerebrospinal fluid Thus it consisted of the following substances NaCl 750 mg $CaCl_2$ 16 6 mg
Na lactate 21 0 mg glucose 58 0 mg and
nals received 2 meq/l K in the perfusion

of the animals were followed through out

Histological preparation

All the animals were perfusion was stopped The brains still containing the perfusion
to check the position
10 % formalin for 2
Fuchsin (Landing *et a*
the controls and experiments simultaneously
hypophysis and the subcommissural organ were estimated as follows The animals were given arbitrary scores ranging from one to five according to the secretory material present in the posterior hypophysis and similarly in the SCO The estimators were not aware of the experimental grouping



Fig 2 Perfusion of rat CSF system The head of the anesthetized rat is fixed with ear pins a a nose ring The fluid runs into the lateral ventricle and drops freely out from the needle serted subarachnoidally beneath the occipital bone

Results and Discussion

at perfusion

Raising the osmolarity of the perfusion fluid had no clear effect on the amount secretory material in the neurohypophysis although the lowest scores were found the group receiving 1 8 % NaCl solution (Table I Fig 3) The corresponding ur group showed no changes although urea is known to diffuse rapidly in the bra (Kleeman *et al* 1962) and was thus expected to have an effect When potassiu was added to the perfusion fluid to give a concentration of 8 meq/l K in the sol tion a marked decrease in the amount of aldehyde-fuchsin-positive material w noted in the SCO (Table I Fig 4) The amount of material in the hypophy remained at the control level

Rat perfusion

Hyperkalemic perfusion fluid effected a similar marked decrease in the amount aldehyde-fuchsin positive material in the SCO as in the experiment with ca (Table I, Fig 4) The group that had only 2 meq/l K in the perfusion fluid h slightly greater amount of stainable material in the SCO In contrast to the ca there was a decrease in the neurohypophyseal secretory material in the group pe fused with artificial fluid containing the normal potassium concentration or 5 meq K^{+} (Fig 3)

The ionic change hyperkalemia thus induced a marked alteration in the conte of secretory material in the SCO of both species and this finding supports the theo of the relation of SCO to ionic balance (cf Palkovits 1965 The decrease in th amount of secretory material in the hypophysis of the rat control group (Group 2 remains enigmatic